MECHANISMS OF ACTIVATION OF GLYCOGENOLYSIS DURING DEVELOPMENT OF MALIGNANT HYPERTHERMIA IN SWINE

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by

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To my husband, M.J.

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ABBREVIATIONS

ENZYMES

ALD	Aldolase
CPK	Creatine phosphokinase
EN	Enolase
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GDH	Glycerol-1-phosphate dehydrogenase or α-Glycerophosphate dehydrogenase
G6P-DH	Glucose-6-phosphate dehydrogenase
HK	Hexokinase
LDH	Lactic dehydrogenase
MDH	Malic dehydrogenase
MK	Myokinase
PGI	Phosphoglucose isomerase
PGluM	Phosphoglucomutase
PGM	Phosphoglycerate mutase
PK	Pyruvate kinase
TIM	Triosephosphate isomerase

CO-ENZYMES AND SUBSTRATES

ADP	Adenosine-5'-diphosphoric acid
AMP	Adenosine-5'-monophosphoric acid

ATP	Adenosine-5'-triphosphoric acid
CP	Creatine phosphate
CMP	Cytidine monophosphate
CDP	Cytidine diphosphate
CTP	Cytidine triphosphate
DAP	Dihydroxyacetone phosphate
FDP	Fructose-1,6-diphosphate
F-1-P	Fructose-1-phosphate
F-6-P	Fructose-6-phosphate
GAP	Glyceraldehyde-3-phosphate
α-GP	Glycerol-1-phosphate
G-1-P	Glucose-1-phosphate
G-6-P	Glucose-6-phosphate
GMP	Guanosine monophosphate
GDP	Guanosine diphosphate
GTP	Guanosine triphosphate
IMP	Inosine monophosphate
IDP	Inosine diphosphate
ITP	Inosine triphosphate
NAD	Nicotinamide-adenine dinucleotide
NADH	Nicotinamide-adenine dinucleotide, reduced
NADP	Nicotinamide-adenine dinucleotide phosphate
NADPH	Nicotinamide-adenine dinucleotide phosphate, reduced
PEP	Phosphoenolpyruvate

2-PG	2-Phosphoglycerate
3-PG	3-Phosphoglycerate
2,3-diPG	2,3-Diphosphoglycerate
P_i	Inorganic phosphate
PYR	Pyruvate
UMP	Uridine monophosphate
UDP	Uridine diphosphate
UTP	Uridine triphosphate

GENERAL

DTNB	5'-5'-dithiobis(2-nitrobenzoic acid)
EDTA	Ethylenediaminetetraacetic acid
KAF	Kinase activating factor
p-CMB	para-chloromercuribenzoate
PCA	Perchloric acid
PSE	Pale, soft, exudative
SR	Sarcoplasmic Reticulum
TCA	Trichloroacetic acid
UDPG	Uridine diphosphate glucose

PART I

INTRODUCTION

I. INTRODUCTION

The syndrome of Malignant Hyperpyrexia in man follows administration of certain general anaesthetic agents, and, although rare, is fatal in 70% of cases (EDITORIAL, 1968). Following exposure to the anaesthetic, there is, in most instances of susceptible individuals, a rapid rise in body temperature, usually within a period of 10 minutes, often accompanied by muscular rigidity of the limbs. Sometimes hyperthermia has been delayed for hours and muscular rigidity not pronounced. The temperature reached maybe 43°C (115°F) or even somewhat above this. Halothane, $CF_3CHBrCl$, a halogenated hydrocarbon, is thought to be responsible for most cases (WILSON, NICHOLS, DENT and ALLEN, 1966). Succinyl choline $\left[\begin{matrix}CH_2COOCH_2CH_2\overset{+}{N}(CH_3)_3\\ |\\ CH_2COOCH_2CH_2\overset{+}{N}(CH_3)_3\end{matrix}\right] 2Cl^-$ a skeletal muscle relaxant employed during anaesthesia, has also been implicated (BRITT and KALOW, 1970; HARRISON, 1971).

A similar syndrome has been described in a certain strain of Landrace pig (HARRISON, BIEBUYCK, TERBLANCHE, DENT, HICKMAN and SAUNDERS, 1968). Malignant Hyperpyrexia in pigs, when induced by halothane or succinyl choline, is indistinguishable from the human conditions and is accompanied by

(1) increase in body temperature to 42-45°C
(2) a severe systemic lactacidosis
(3) stiffness or rigor of skeletal muscle and
(4) changes in the water and electrolyte metabolism (BERMAN, HARRISON, BULL and KENCH, 1970).

The present study was undertaken to discover whether the severe lactacidosis found in these animals was due to activation of the rate-limiting enzymes in the glycolytic pathway in skeletal muscle of affected animals, and also to investigate the possible mechanism by which halothane or succinyl choline might stimulate enzymes or provoke the syndrome of hyperthermia. More specifically, our intention was to identify the step or steps of the glycolytic pathway which become activated and result in massive release of lactic acid in

skeletal muscle. Possible mechanisms by which halothane or succinyl choline could stimulate the enzymes concerned would be explored. Previous studies have not implicated anoxia as a factor stimulating glycolysis in this condition (BERMAN, HARRISON, BULL and KENCH, 1970).

II. THE CONTROL OF GLYCOGEN METABOLISM IN MUSCLE.

A. THE EMBDEN-MEYERHOF PATHWAY

This pathway may be considered to comprise those steps by which carbohydrate substrates are metabolised anaerobically to pyruvate (Fig. 1 and 2). The initial substrate, either glycogen or glucose, enters the pathway by phosphorylation, either by inorganic phosphate (phosphorolysis of glycogen) or by ATP (hexokinase reaction for glucose). An additional phosphorylation is involved with the consumption of ATP during the conversion of fructose-6-phosphate to fructose-1,6-

FIG. 1

THE EMBDEN-MEYERHOF PATHWAY

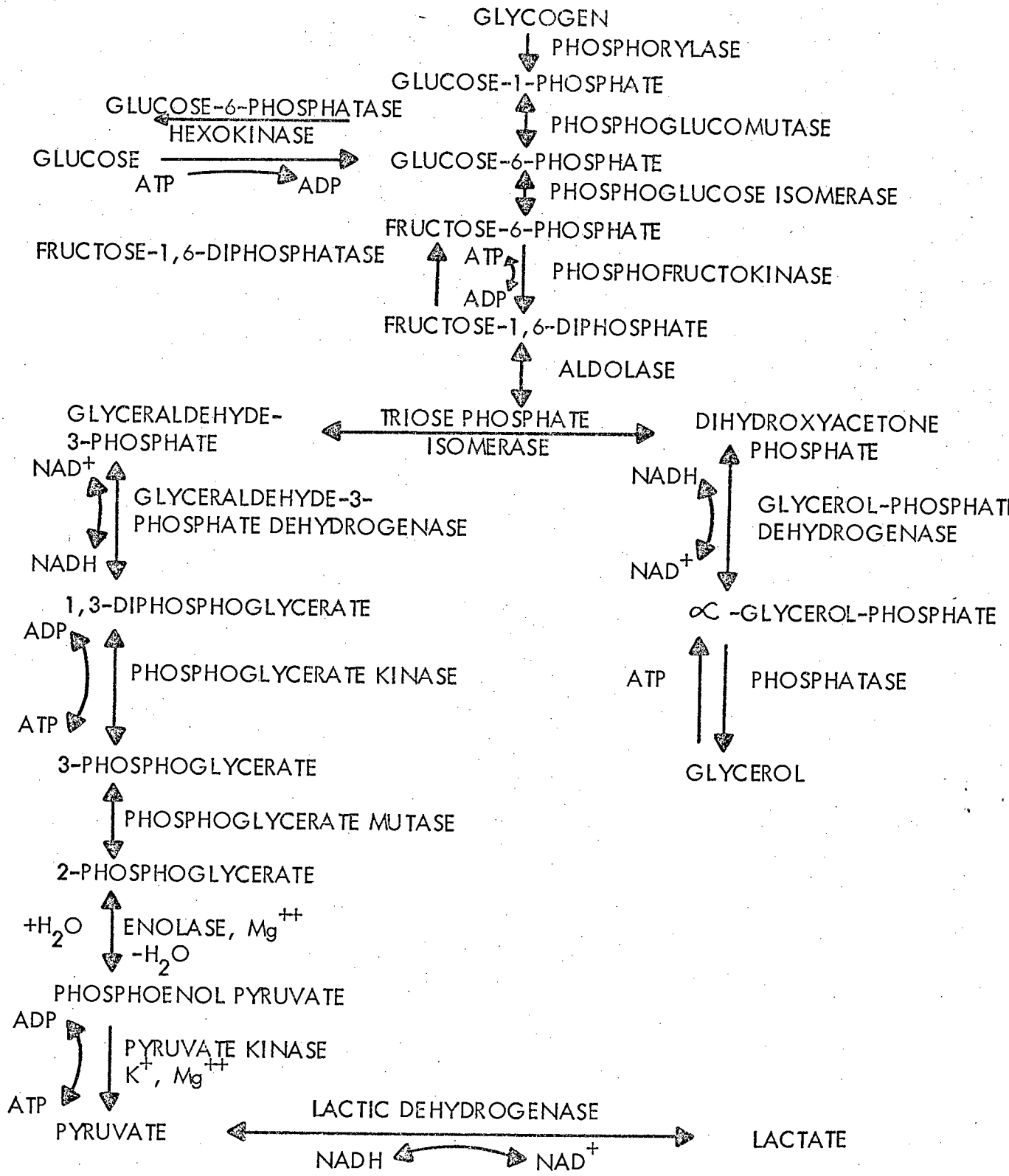
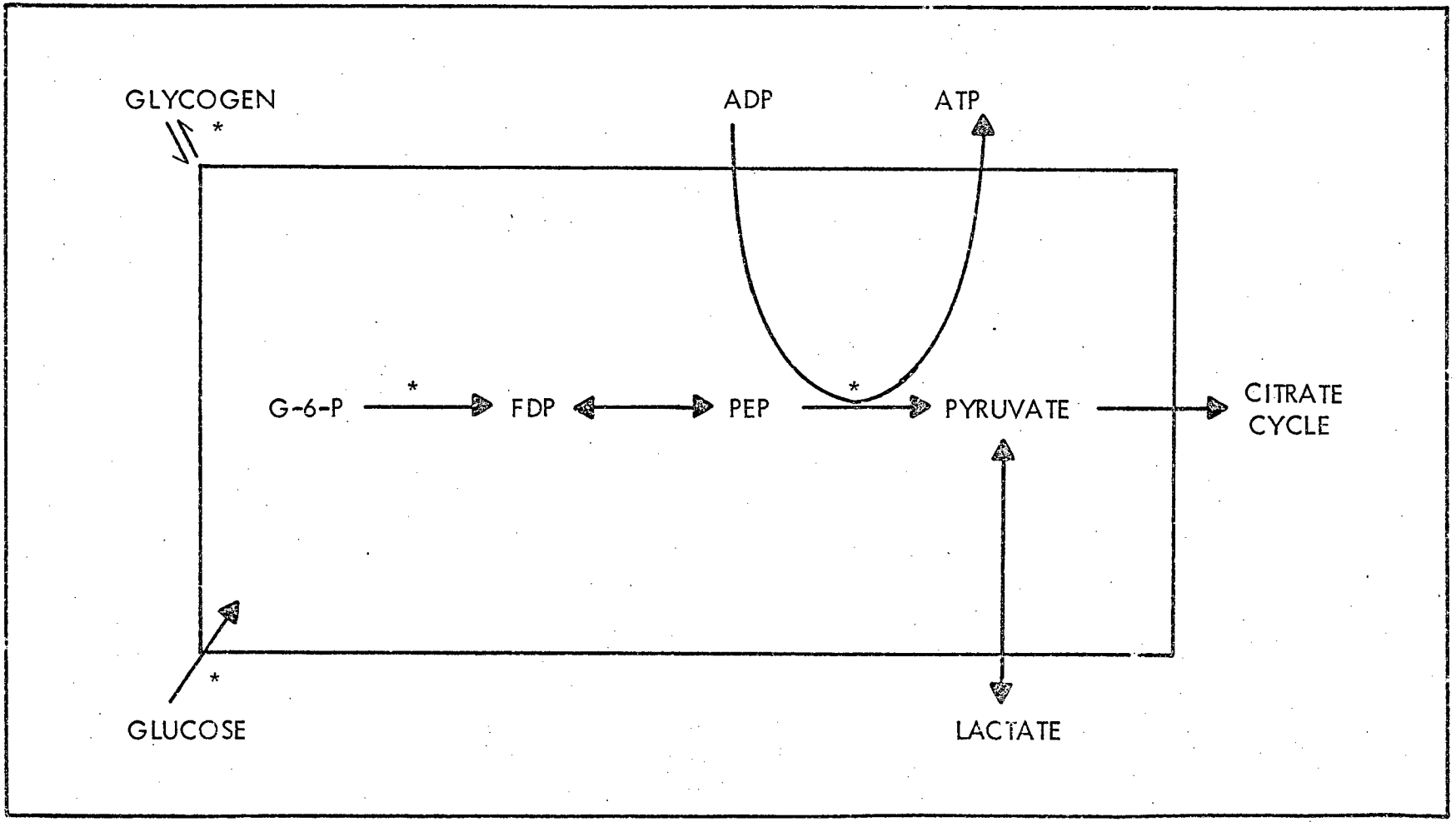


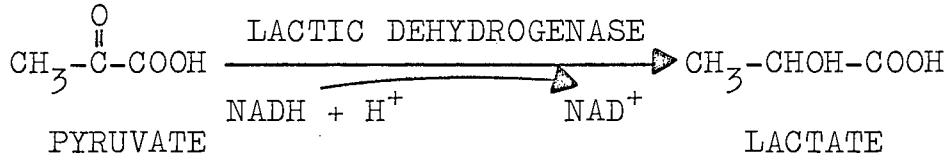
FIG. 2

CONTROL OF GLYCOLYSIS



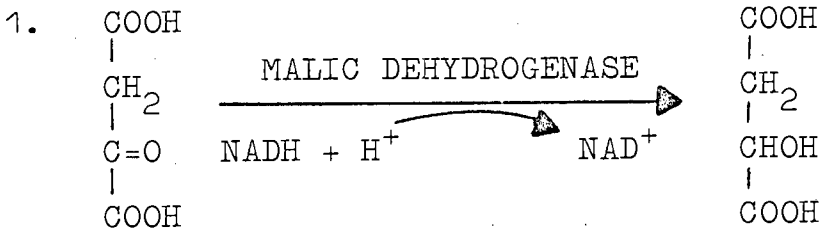
* CONTROL POINTS.

diphosphate. Each of these phosphorylation steps appears to function as a control point for the regulation of the rates of glycolysis and glycogenolysis. The rate of flux along the pathway will be influenced by the availability of certain cofactors. Thus, lack of ADP will inhibit the pyruvate kinase reaction and, similarly, insufficient NAD^+ will impede the conversion of glyceraldehyde-3-phosphate to 3-phosphoglycerate, as catalysed by glyceraldehyde-3-phosphate dehydrogenase. The regeneration of NAD^+ by the conversion of pyruvate to lactate



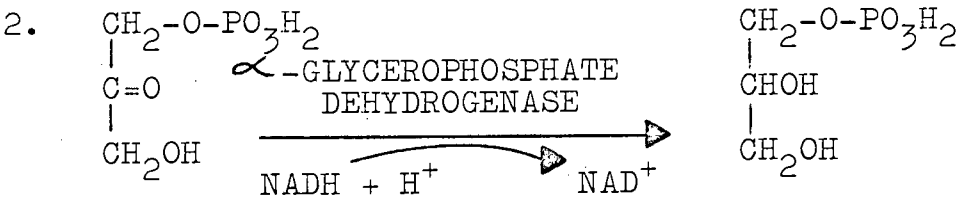
allows glycolysis to continue under anaerobic conditions, and is the reason for lactate accumulation during rapid glycolysis. Two other systems could be affected by NADH accumulation, namely:

1.



OXALOACETATE MALATE

2.



DIHYDROXYACETONE PHOSPHATE ∝-GLYCEROPHOSPHATE

In the following discussion, steps known to be control points of glycogenolysis will be considered in most detail. When Malignant Hyperpyrexia was induced in whole living animals, with the development of lactacidosis blood glucose concentration did not fall, but rose significantly. It was inferred that glycogen was probably the sole source of lactate. The hexokinase reaction will not, therefore, be further considered.

B. GLYCOGEN SYNTHESIS IN MUSCLE

Synthesis of glycogen *in vitro* by a purified enzyme was achieved by CORI, SCHMIDT and CORI, 1939. It was generally accepted for a long time that phosphorylase *in vivo* was responsible for the synthesis, as well as for the breakdown of glycogen, in view of the fact that the phosphorylase reaction is reversible *in vitro* and can form glycogen.

$$\text{GLUCOSE-1-PHOSPHATE} + \text{GLYCOGEN}_n \underset{\longleftarrow}{\xrightarrow{\text{PHOSPHORYLASE}}} \text{GLYCOGEN}_{(n+1)} + \text{INORGANIC PHOSPHATE}$$

(CORI, CORI and GREEN, 1943).

However, demonstration of the absence of phosphorylase in glycogen storage disease of muscle in man, McArdle's Syndrome (McARDLE, 1951) was a strong indication that glycogen synthesis and catabolism are independent processes. This is now clearly established, in addition to the reciprocal actions of inhibitors and stimulators of these independent pathways.

The discovery of an enzyme in liver, uridine diphosphate glucose-glycogen-transglucosylase (glycogen synthetase), which is responsible for the transfer of glucose from uridine diphosphate glucose (UDPG) to glycogen proved that phosphorylase plays no significant role in glycogen synthesis (LELOIR and CARDINI, 1957).

Synthesis of glycogen from glucose is presently envisaged as follows:

1. $$\text{GLUCOSE} + \text{ATP} \xrightarrow[Mg^{++}]{\text{HEXOKINASE}} \text{GLUCOSE-6-PHOSPHATE} + \text{ADP}$$

2. $$\text{GLUCOSE-6-PHOSPHATE} \underset{\text{GLUCOSE-1-PHOSPHATE}}{\overset{\text{PHOSPHOGLUCOMUTASE}}{\rightleftharpoons}}$$

3. $$\text{URIDINE TRIPHOSPHATE} + \text{GLUCOSE-1-PHOSPHATE} \xrightarrow[\text{PYROPHOSPHORYLASE}]{\text{UDPG}} \text{UDPG} + \text{PYROPHOSPHATE}$$

4. $$\text{URIDINE DIPHOSPHATE GLUCOSE} + \text{PRIMER} \xrightarrow[\text{SYNTHETASE}]{\text{GLYCOGEN}} \text{GLYCOGEN} + \text{URIDINE DIPHOSPHATE}$$

(i) Properties of Glycogen Synthetase

Glycogen synthetase in muscle exists in two interconvertible forms, one of which is dependent on glucose-6-phosphate for activity. This form has been called the dependent or D form. The other form is active in the absence of glucose-6-phosphate and has been called the independent or I form.

The I and D forms have been found in skeletal muscle of rat (ROSELL-PEREZ, VILLAR-PALASI and LARNER, 1962), lamb (TRAUT and LIPMAN, 1963), rabbit and dog (ROSELL-PEREZ and LARNER, 1964 (a) and (b)), in the fatbody of the silkmoth (MURPHY and WYATT, 1965), mature human erythrocytes and leucocytes (CORNBLATH, STEINER, BRYAN and KING, 1965) and in normal human lymphocytes (HEDESKOV, ESMANN and ROSELL-PEREZ, 1966).

Conversion of the I to the D form requires ATP and Mg^{++}. The change is catalyzed by transferase I kinase and involves the transfer of the terminal phosphate from ATP to the enzyme in which a serine residue is phosphorylated. The reverse conversion of the D to the I form is catalyzed by transferase D phosphatase, and

is accompanied by loss of inorganic phosphate from the enzyme.

(1) GLYCOGEN SYNTHETASE I + nATP $\xrightarrow[Mg^{++}]{\text{TRANSFERASE I KINASE}}$ GLYCOGEN SYNTHETASE D + nADP

(2) GLYCOGEN SYNTHETASE D $\xrightarrow{\text{TRANSFERASE D PHOSPHATASE}}$ GLYCOGEN SYNTHETASE I + nINORGANIC PHOSPHATE

(FRIEDMAN and LARNER, 1963).

Purified rabbit skeletal muscle phosphorylase b kinase and activated phosphorylase b kinase, produced by incubation with cyclic AMP and ATP, had no significant effect upon glycogen synthetase I as substrate. Therefore the kinase responsible for the phosphorylation of glycogen synthetase I and phosphorylase b are different enzymes (FRIEDMAN and LARNER, 1965)

The action of Ca^{++} ions on the conversion of glycogen synthetase I to D form in rat skeletal muscle has also been investigated. Ca^{++} promotes the I to D conversion in muscle. This change does not involve ATP, and is not

affected by cyclic AMP. It requires, however, a protein factor which is very similar to that involved in the activation of inactive phosphorylase b kinase by Ca^{++} (BELOCOPITOW, APPLEMAN and TORRES, 1965).

The properties of glycogen synthetase D, as prepared by incubation with Ca^{++} or with ATP have been compared. The two preparations exhibited different sensitivity to the action of heat and trypsin. Glycogen synthetase D formed in the presence of Ca^{++} is more sensitive to heat treatment and less stable on storage at $-20^{\circ}C$ than glycogen synthetase D generated with ATP. The glycogen synthetase D obtained with ATP is reconvertible to the I form, while that prepared with Ca^{++} is not (BELOCOPITOW, FERNANDEZ, BIRNBAUMER and TORRES, 1967). These observations suggest that Ca^{++} may not be the physiological inactivator of this system.

ATP and ADP are potent inhibitors for both forms of the enzyme isolated from rat skeletal muscle but AMP and inorganic phosphate cause much less inhibition. Addition of

glucose-6-phosphate reverses this inhibition of ATP, ADP, AMP and inorganic phosphate to a great extent. However, the inhibition caused by UDPG, a substrate analogue, is not relieved by the addition of glucose-6-phosphate (PIRAS, ROTHMAN and CABIB, 1967).

The effect of glycogen on the transferase D phosphatase activity in isolated perfused rat heart has also been studied. Transferase D phosphatase, which abets the conversion of the D to I form, was shown to be inhibited by glycogen in vitro (HUIJING, NUTTALL, VILLAR-PALASI and LARNER, 1969).

The relative distribution of glycogen synthetase as I or D in mouse skeletal muscle and in isolated intact rat diaphragm is related to the tissue concentration of glycogen. Glycogen synthesis is thus regulated by an unknown mechanism which influences the interconversion of glycogen synthetase I to D in such a way that it favours glycogen synthesis when the tissue concentration of the polysaccharide is low, and decreases glycogen synthesis when its concentration is high.

Presumably, inhibition of transferase D phosphatase by glycogen arises as a consequence of an allosteric conformational change in the active site of the enzyme, induced through interaction of the polysaccharide with a region of the enzyme molecule in the neighbourhood of the active site. Activation of glycogen synthetase D by glucose-6-phosphate could be the manifestation of an allosteric modification of the tertiary structure of the enzyme.

Insulin stimulates glycogen synthesis in isolated rat diaphragm (DANFORTH, 1965). The in vivo effect of insulin on muscle glycogen synthetase activity of rat muscle has been investigated (VILLAR-PALASI and WENGER, 1967). Five minutes after the injection of rats with insulin, glycogen synthetase I activity had increased by 60% and after 10 minutes had risen by 90%. There was, however, no change in the total glycogen synthetase activity (that is, I and D combined) or in the glycogen content of the muscle. Insulin had no influence on transferase D phosphatase activity which converts the D to I form, but caused a marked decline

in transferase I kinase activity which catalyzes the I to D conversion. The fall in transferase I kinase activity of rat muscle extracts following administration of insulin was rectified by the addition of cyclic AMP.

It is assumed, therefore, that there are two glycogen synthetase kinases, one dependent on cyclic AMP for activity, and the other independent of cyclic AMP, both catalyzing the conversion of the I to D form.

C. GLYCOGEN DEGRADATION

The complete degradation of glycogen in animal tissues requires two enzymes:

(a) Phosphorylase, for the phosphorolysis, through the action of inorganic orthophosphate, on the α-1:4-linkages between glucose residues of unbranched portions of the glycogen molecule. Glucose residues are removed singly and progressively from the outer exposed ends of glycogen until a 1:4 - 1:6 branch is reached. From each

glucose residue thus "whittled away" one molecule of glucose-1-phosphate is formed.

(b) Amylo-1,4 - 1,6 transglucosidase, also called the debranching enzyme, which facilitates hydrolysis of the 1,6 glucosidic linkage at each branch point, with the production of one molecule of free glucose (CORI and LARNER, 1951.

(i) Skeletal Muscle Phosphorylase

Phosphorylase exists in skeletal muscle in two forms that have been designated phosphorylase a and phosphorylase b.

Phosphorylase a, also known as the active form, possesses 60 - 70% of its maximal activity in the absence of AMP. Phosphorylase b, known also as the inactive form, has an absolute requirement for AMP. In the presence of AMP both phosphorylase a and b have the same activity per mg. of protein (CORI and GREEN, 1943).

Isolation and crystallization of rabbit skeletal muscle phosphorylase was achieved by GREEN and CORI, 1943. Resting rabbit skeletal muscle contains phosphorylase predominantly in

the b form (KREBS and FISCHER, 1955). Phosphorylases have been isolated and crystallized from a number of tissues; phosphorylase b from rabbit skeletal muscle (FISCHER and KREBS, 1958), both a and b forms from human autopsy skeletal muscle (YUNIS, FISCHER and KREBS, 1960), frog skeletal muscle (METZGER, GLASER and HELMREICH, 1968), human blood platelets (KARPATKIN and LANGER, 1969), liver (SUTHERLAND and WOSILAIT, 1956), rat and beef kidneys (VILLAR-PALASI and GAZQUEZ-MARTINEZ, 1968) and from lobster muscle (COWGILL, 1959).

The earlier determinations of the size of crystalline rabbit muscle phosphorylase revealed a molecule with a molecular weight between 340 000 and 400 000 daltons (ONCLEY, 1943). It was established later that rabbit skeletal muscle phosphorylase a and b have a molecular weight of 495 000 and 242 000 daltons respectively, i.e. phosphorylase b has half the molecular weight of phosphorylase a (KELLER and CORI, 1953). A reinvestigation into the molecular weight of phosphorylase a and b has given values of 370 000 and 185 000 respectively (SEERY, FISCHER and TELLER, 1967).

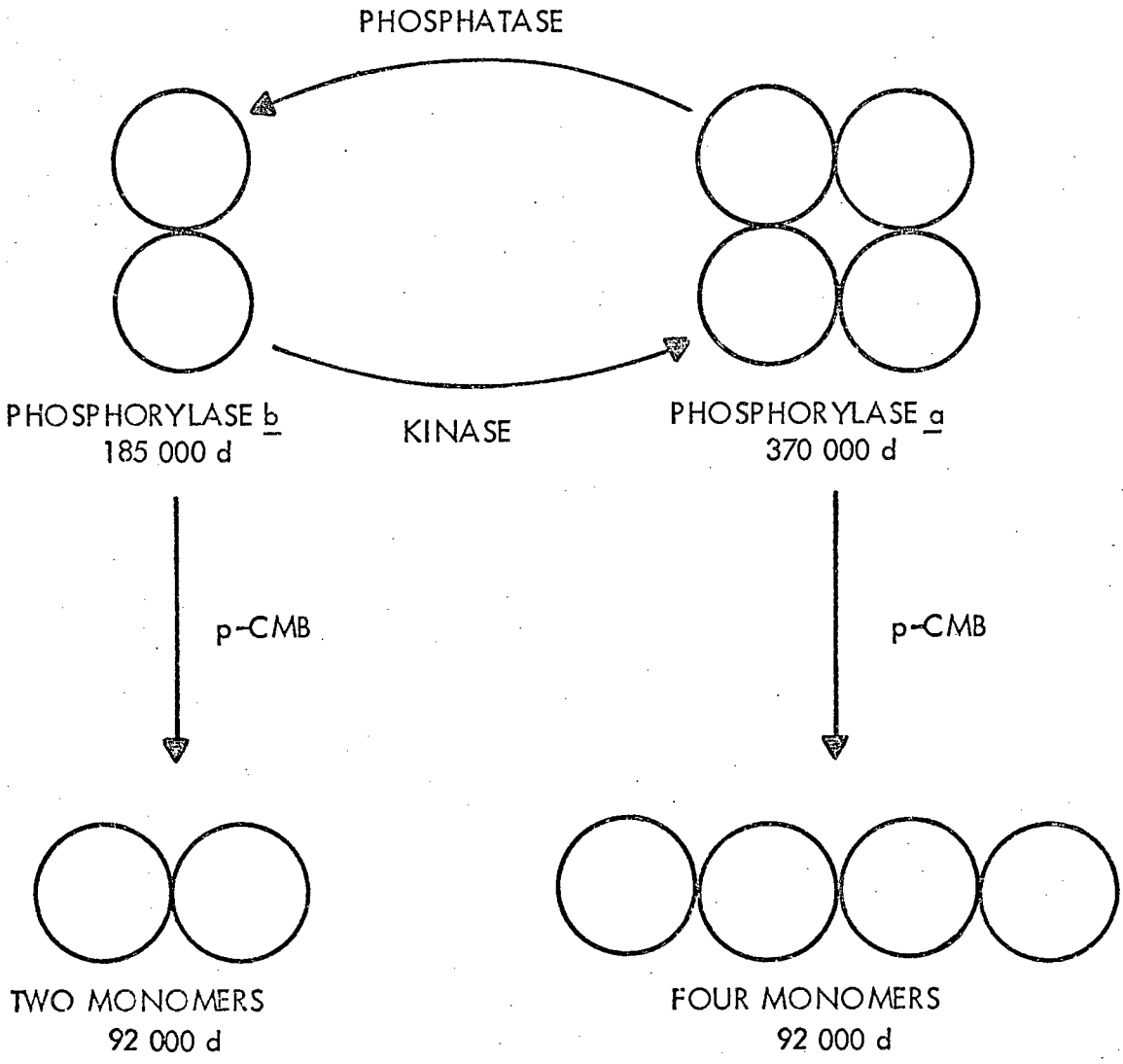
Phosphorylase a and b can also be further dissociated into subunits or monomers by treatment with certain mercurial compounds, such as parachloromercuribenzoate (p-CMB). Each subunit has a molecular weight of 92 000, which is in agreement with the more recent determinations of native a and b forms. Phosphorylase a, which is a tetramer, gives four phosphorylated subunits of equal molecular weight and phosphorylase b, which is a dimer, produces two non-phosphorylated subunits of equal molecular weights when treated with p-CMB (MADSEN and CORI, 1956) (Fig. 3)

The determination of the amino acid composition of rabbit muscle phosphorylase, using microbiological, isotopic and spectrophotometric techniques, was achieved by VELICK and WICKS, (1951). The amino acid compositions of frog, rabbit and human skeletal muscle phosphorylases are quite similar, but the frog and rabbit skeletal muscle phosphorylases differ immunologically (METZGER, GLASER and HELMREICH, 1968).

The presence of a vitamin B_6 derivative as a component of phosphorylase was first reported by VELICK and WICKS (1951). Phosphorylase a

FIG. 3.

AGGREGATION-DISSOCIATION OF GLYCOGEN PHOSPHORYLASE SUBUNITS



(FISCHER AND KREBS, 1966)

contains eight organic phosphate groups per mole or two phosphate groups per sub-unit with a molecular weight of 125 000 daltons. Four of these phosphate groups could be extracted by precipitation of the enzyme with trichloracetic acid (TCA) or perchloric acid (PCA). The TCA or PCA-soluble compound was isolated and identified as pyridoxal-5'-phosphate (BARANOWSKI, ILLINGWORTH, BROWN and CORI, 1957). Rabbit muscle phosphorylase a and b contain four and two moles respectively of firmly bound pyridoxal-5'-phosphate per mole of enzyme and the enzyme is inactive after the removal of pyridoxal-5'-phosphate. On incubation of the enzyme with quantities of pyridoxal-5'-phosphate slightly greater than those removed from the enzyme, reactivation occurs. Pyridoxamine-5'-phosphate and free pyridoxal cannot replace pyridoxal-5'-phosphate (CORI and ILLINGWORTH, 1957). Pyridoxal-5'-phosphate has also been reported to be present in phosphorylase of cat muscle (KENT, KREBS and FISCHER, 1958), of frog skeletal muscle (METZGER, GLASER and HELMREICH, 1968) and likewise in the enzyme of human muscle (YUNIS,

FISCHER and KREBS, 1960).

The function of bound B_6 cannot as yet be explained although it is obviously necessary for enzyme activity. No mechanism comparable to its decarboxylating function in the pyruvate or α-oxoglutarate dehydrogenase systems or its role in transamination can be imagined. Perhaps its salient importance relates to stabilization of the quaternary structure of the enzyme, as a tetramer requisite for its catalytic activity.

Phosphorylase a and b bind four and two moles of AMP per mole of protein respectively. Phosphorylase a has four binding sites for this nucleotide, one on each subunit, with a molar dissocation constant of the complex of 1.5×10^{-6}. Phosphorylase b has two binding sites for AMP, one on each subunit, with a molar dissociation constant of 50×10^{-6} MADSEN and CORI, 1957). The lower affinity of the b form for AMP, an essential cofactor for activity, is relevant to control of phosphorylase activity *in vivo*.

It is now well established that phosphorylase is an "SH-enzyme", i.e. an enzyme whose

activity is dependent on the presence of one or more thiol (-SH) groups. Reagents which react with the thiol functions para-chloromercuribenzoate (MADSEN and CORI, 1956; MADSEN and GURD, 1956; KUDO and SHUKUYA, 1964), N-ethylmaleimide (KUDO and SHUKUYA, 1964), iodoacetoamide (BATTELL, ZARKADAS, SMILLIE and MADSEN, 1968), 2,4-dinitro-1-fluorobenzene(PHILIP and GRAVES, 1968) and 5,5'-dithiobis(2-nitrobenzoic acid) (KLEPPE and DAMJANOVICH, 1969) can inhibit activity completely. The -SH groups do not appear to participate in the aggregation-dissociation phenomena.

The activation of phosphorylase b by AMP is stimulated by the polyamines, spermine ($H_2N-(CH_2)_3-NH-(CH_2)_4-NH-(CH_2)_3-NH_2$), spermidine ($H_2N-(CH_2)_4-NH-(CH_2)_3-NH_2$) and putrescine ($H_2N-(CH_2)_5-NH_2$). Spermine and spermidine have been found in relative abundance in preparations of pH 5 enzymes from rat brain, and may play a role in microsomal protein synthesis (GIORGI, 1970). Stimulation of phosphorylase b by polyamines is more pronounced at low AMP concentrations. When

phosphorylase a is incubated with trypsin, it is converted into yet another active phosphorylase, phosphorylase b'.

$$\text{PHOSPHORYLASE a} \xrightarrow{\text{TRYPSIN}} \text{PHOSPHORYLASE b'}$$

Phosphorylases a and b' are not significantly activated by spermine at either high or low AMP concentrations, nor is phosphorylase a activated significantly by spermine in the absence of AMP. Spermine was found to be the most effective of the polyamines in the activation of phosphorylase b, whilst putrescine was the least effective (WANG, HUMNISKI and BLACK, 1968).

Crystalline rabbit muscle phosphorylase a, in the absence as well as in the presence of AMP, is inhibited by very small amounts (μgm) of salmine, but, paradoxically, the extent of inhibition falls as the salmine concentration is increased.

The affinity of phosphorylase b for AMP and IMP is greatly augmented in the presence of salmine. Salmine, however, has no effect

on the activity of phosphorylase b', produced by the action of trypsin (KREBS, 1954). The exact significance of the effects of polyamines remains obscure.

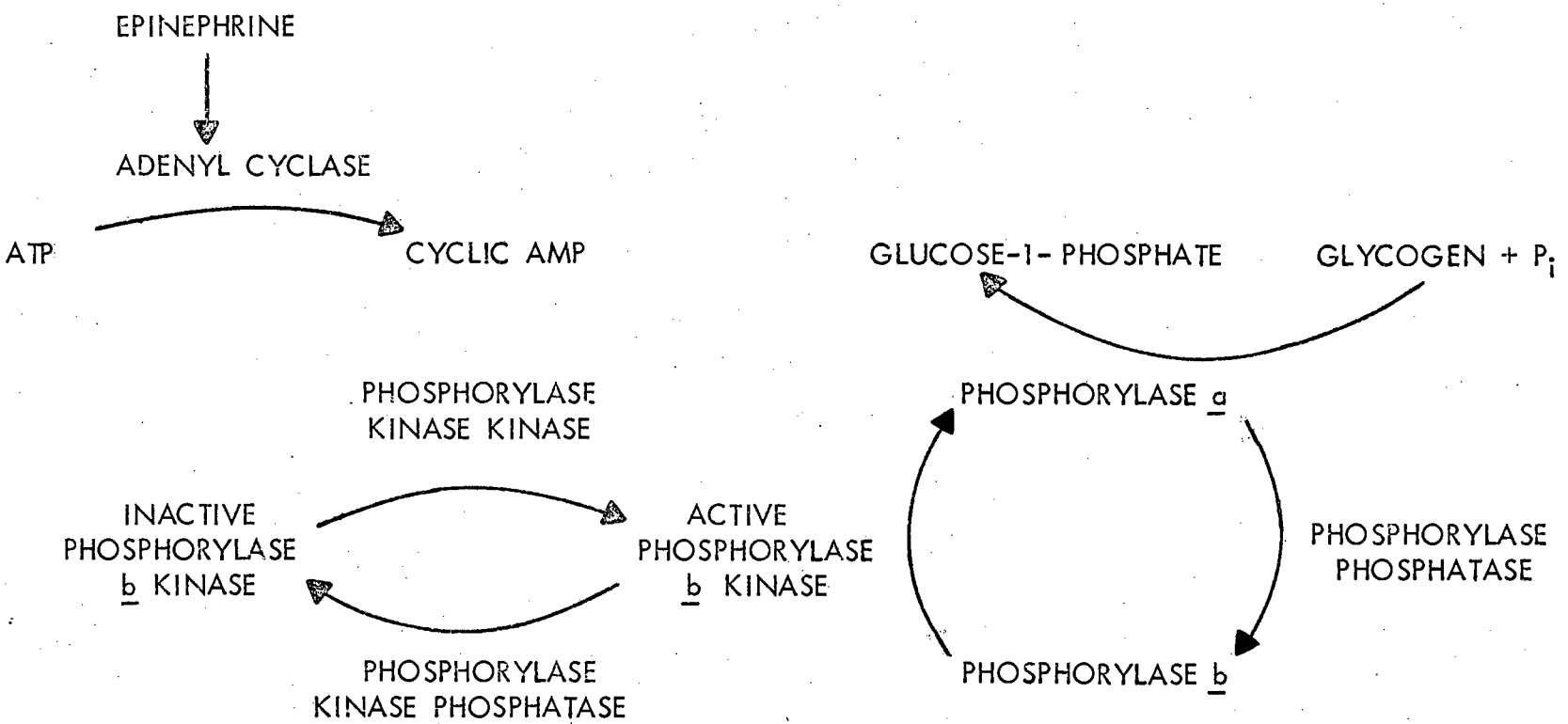
Inhibition of phosphorylase b by glucose-6-phosphate is partially competitive with respect to AMP, an activator (WANG, TU and LO, 1970). Interaction between the activator and inhibitor of phosphorylase b is allosteric in nature, a negative heterotropic interaction. A negative heterotropic interaction is one in which conformational changes are induced, by the inhibitor, at the binding site in the enzyme for substrate or activator. In the presence of 20mM glucose-6-phosphate, the affinity of the enzyme for the activator AMP was lowered 10-18 fold.

(ii) The conversion of phosphorylase b to phosphorylase a.

The conversion of phosphorylase b to phosphorylase a is depicted in Fig. 4.

FIG. 4

CONTROL OF GLYCOGEN PHOSPHORYLASE ACTIVITY



The change of rabbit skeletal muscle phosphorylase b to a, catalyzed by an enzyme which requires ATP and Mg^{++} has been studied by FISCHER and KREBS (1955). Experiments with γ-^{32}P-labelled ATP revealed that phosphate was transferred from the terminal phosphate of ATP to phosphorylase a (KREBS and FISCHER, 1956). It was established that four moles of phosphate are incorporated per mole of phosphorylase a and four moles of ADP are formed.

$$2\ \text{PHOSPHORYLASE b} + 4\text{ATP} \xrightarrow[\text{b KINASE}]{\text{PHOSPHORYLASE}} \text{PHOSPHORYLASE a} + 4\text{ADP}$$

Krebs and Fischer proposed that the enzyme catalyzing this reaction be called phosphorylase b kinase, since it involved the transfer of the terminal phosphate of ATP. This reaction was later shown to be irreversible (KREBS, KENT and FISCHER, 1958). A serine residue in phosphorylase b is the acceptor of phosphate, and the primary amino

acid sequence in the site has the following structure; LYS-GLU.NH_2-ILEU-SER.P-VAL-ARG (FISCHER, GRAVES, CRITTENDEN and KREBS, 1959). Rabbit skeletal muscle phosphorylase b kinase has been prepared as a nearly homogeneous protein (DE LANGE, KEMP, RILEY, COOPER and KREBS, 1968).

When extracted from rabbit skeletal muscle, part of the phosphorylase b kinase so obtained is inactive when assayed at pH 6,8 or below and is referred to as the non-active phosphorylase b kinase. Above this pH, the enzyme is active, reaching a maximum at pH 8,2, and is called active phosphorylase b kinase. The ratio of activity at pH 6,8 and pH 8,2 is employed as an index of the relative activity of the kinase (KREBS, GRAVES and FISCHER, 1959). A rise in the pH 6,8 to 8,2 ratio was demonstrated in isolated muscle enzyme systems (POSNER, HAMMERMEISTER, BRATVOLD and KREBS, 1964) and also after the administration of epinephrine to frog and rat muscle (POSNER, STERN and KREBS, 1965; DRUMMOND, HARWOOD and POWELL, 1969), in perfused rat hearts (DRUMMOND, DUNCAN and HERTZMAN, 1966;

HAMMERMEISTER, YUNIS and KREBS, 1965) and in dog hearts (NAMM and MAYER, 1967).

Activation of isolated non-active phosphorylase b kinase can be achieved in various ways, namely (a) by incubation with trypsin (b) by addition of Mg^{++}, ATP and cyclic AMP or (c) of Ca^{++}. Low concentrations of trypsin cause powerful activation of purified muscle phosphorylase b kinase, with a rise in enzyme activity at pH 6,8 and at pH 8,2 (KREBS, LOVE, BRATVOLD, TRAYSER, MEYER and FISCHER, 1964). Preincubation with ATP and Mg^{++} ions activates rabbit skeletal phosphorylase b kinase and although this activation is enhanced by the addition of cyclic AMP, it does not appear to be essential (KREBS, GRAVES and FISCHER, 1959; KREBS, LOVE, BRATVOLD, TRAYSER, MEYER and FISCHER, 1964). The same observations were made with bovine heart phosphorylase b kinase (DRUMMOND, DUNCAN and FRIESEN, 1965; DRUMMOND and DUNCAN, 1966; HAMMERMEISTER, YUNIS and KREBS, 1965) and the human muscle enzyme (HANABUSA and KOBAYASHI, 1967).

Activation by ATP was found to be specific. No activation took place with any of the following nucleotides tested together with Mg^{++}: 5'-AMP, 3'-AMP, cyclic 3',5'-AMP, CMP, CDP, CTP, UMP, UDP, UTP, GMP, GDP, GTP, IMP, IDP and ITP (KREBS, LOVE, BRATVOLD, TRAYSER, MEYER and FISCHER, 1964).

A protein kinase, which is cyclic AMP-dependent has been purified from rabbit brain (MIYAMOTO, JUO and GREENGARD, 1969) and rabbit skeletal muscle (WALSH, PERKINS and KREBS, 1968). This protein kinase phosphorylates proteins such as casein and protamine, and also expedites the rate of activation and phosphorylation of phosphorylase b kinase by ATP and Mg^{++} ions. Since the activity of this kinase is entirely dependent on cyclic AMP, it seems likely that this enzyme is involved during epinephrine stimulation of adenyl cyclase, which catalyzes the conversion of ATP to cyclic AMP, and in turn activates phosphorylase b kinase. The proposed name for this protein kinase is phosphorylase kinase kinase (WALSH, PERKINS and KREBS, 1968). The phosphorylation-dephosphorylation process which activates phosphorylase b kinase is of physiological importance since it is reversible.

Non-activated phosphorylase b kinase can be activated by incubation with Ca^{++}-ions. This activation is not reversible. In the phosphorylase b to a reaction itself, Ca^{++} is a competitive inhibitor with respect to Mg^{++} (KREBS, GRAVES and FISCHER, 1959). Activation of rabbit skeletal muscle phosphorylase b kinase by incubation with Ca^{++} was found to require an additional protein factor, the kinase activating factor (KAF). This process is also not reversible, as by chelation of Ca^{++} with EDTA. Sr^{++} and Ba^{++} were also found to be activators of phosphorylase b kinase. KAF has been purified 700 fold and was then observed to have no proteolytic activity. Purified KAF is very unstable in the presence of Ca^{++}, and about 50% of its activity was lost on incubation at 30°C with 0,015M Ca^{++}. In the presence of EDTA, however, KAF can be stored frozen without loss of activity (MEYER, FISCHER and KREBS, 1964). KAF purified from rabbit skeletal muscle was subsequently observed to possess proteolytic activity (HUSTON and KREBS, 1968). In the presence of Ca^{++}, KAF causes enzyme activation

and release of peptides. This proteolytic action of KAF in the presence of Ca^{++} and the irreversibility of the activation of phosphorylase b kinase makes it unlikely that this mechanism is of any physiological importance.

Nonetheless, OZAWA, HOSOI and EBASI, 1967, remarked that activation of phosphorylase b kinase of rabbit skeletal muscle by Ca^{++} ions alone was reversible. Free Ca^{++} ions at a concentration of 10^{-7}M generated more than half the maximum activity. This observation suggests that phosphorylase b kinase could be stimulated by Ca^{++} ions released from the sarcoplasmic reticulum under physiological conditions.

A kinase inhibitory factor, which prevents activation by Ca^{++}, has been isolated from beef heart and purified. The action of the kinase inhibitory factor appears to be non-catalytic (DRUMMOND and DUNCAN, 1966).

The role of Ca^{++} activation has been studied in a rabbit muscle protein-glycogen complex, containing glycogen granules, and

fragments of the sarcoplasmic reticulum to which are attached phosphorylase, phosphorylase kinase, phosphorylase phosphatase and other enzymes (HEILMEYER, MEYER, HASCHKE and FISCHER, 1970). This fraction contained phosphorylase in its inactive b form, since the phosphorylase kinase present was inactive and phosphorylase phosphatase fully active. Upon the addition of Mg^{++}, ATP and Ca^{++} phosphorylase kinase was activated and phosphorylase b was converted to phosphorylase a. No activation was achieved with Mg^{++} and ATP alone. Depletion of ATP in the system led to reconversion of phosphorylase a to phosphorylase b by phosphorylase phosphatase. On addition of ATP, the whole cycle was repeated, being known as the "flash activation" of phosphorylase. Activation by Ca^{++} was reversible by addition of chelating agents and could not, therefore, be due to proteolytic attack by the calcium-dependent KAF. The concentration of free calcium required for half-maximum activation of phosphorylase kinase in this system was 2×10^{-6}M. For purified systems, the concentration of Ca^{++} needed was 10^{-7}M,

the same concentration that is required to initiate muscle contraction.

The affinity of phosphorylase kinase for phosphorylase b was magnified 13-fold during Ca^{++}-activation. No evidence could be elicited as to whether activation of phosphorylase kinase by Ca^{++} was accompanied by phosphorylation. In the absence of Ca^{++}, and in the presence of Mg^{++}, ATP and cyclic AMP, only slight activation of phosphorylase was observed. The elegant studies of Ebashi and his colleagues appear firmly to have established the physiological role of Ca^{++} as an activator or regulator of glycogenolysis at the phosphorylase step. Ca^{++} is thus the link coupling activation of the contractile mechanism and the breakdown of glycogen which can supply anaerobic energy for such contraction.

Skeletal muscle and other tissues contain a phosphatase that catalyzes the dephosphorylation of activated phosphorylase kinase. This phosphatase, designated as phosphorylase kinase phosphatase, is activated by metal ions and inhibited by sodium fluoride (RILEY, DE LANGE, BRATVOLD and KREBS, 1968).

(iii) The conversion of phosphorylase a to phosphorylase b.

$$\text{PHOSPHORYLASE } \underline{a} + 4H_2O \xrightarrow[\text{PHOSPHATASE}]{\text{PHOSPHORYLASE}} 2 \text{ PHOSPHORYLASE } \underline{b} + 4P_i$$

(GRAVES, FISCHER and KREBS, 1960).

Rabbit skeletal muscle and other tissues contain an enzyme which catalyzes the conversion of phosphorylase a to phosphorylase b (CORI and GREEN, 1943) and the conversion is accompanied by a halving in the molecular weight (KELLER and CORI, 1953). This enzyme was orignally designated "PR-enzyme" because it was thought to function as a prosthetic group-removing enzyme. The PR-enzyme was thought to cleave a prosthetic group from phosphorylase a, which contained AMP (CORI and GREEN, 1943). However attempts to demonstrate that AMP was detached during the conversion of phosphorylase a to b were unsuccessful. There is no evidence that AMP appears among the products (CORI and CORI, 1945; VELICK and WICKS, 1951).

The PR-enzyme was partially purified from rabbit skeletal muscle and some of its properties were determined (CORI and CORI, 1945; KELLER and CORI, 1955). The activaty of this enzyme was boosted by the presence of cysteine, inhibited by sodium fluoride and no stimulation was observed with Mn^{++}, Mg^{++} or Ca^{++} ions. Synthetic substrates of trypsin inhibited the PR-enzyme and it was suggested that it might be a proteolytic enzyme (KELLER and FRIED, 1955).

Later it was observed that change of phosphorylase a into b was accompanied by a release of inorganic phosphate (RALL, WOSILAIT and SUTHERLAND, 1956; FISCHER, GRAVES and KREBS, 1957) and the name phosphorylase phosphatase was proposed for the PR-enzyme (WOSILAIT and SUTHERLAND, 1956).

Phosphorylase a phosphatase activity can be regulated by reversible mechanisms of activation and inactivation. Preincubation of partially purified phosphorylase a phosphatase from bovine adrenal cortex in the presence of ATP and Mg^{++} raises the activity of the enzyme.

Incubation of activated phosphorylase a phosphatase in the presence of ATP inactivated the enzyme. Addition of cyclic AMP, as well as ATP and Mg^{++}, converted the active form into a less active one (MERLEVEDE and RILEY, 1966). Evidence was adduced that a similar mechanism operates in pigeon breast muscle (CHELALA and TORRES, 1969) and in dog liver (MERLEVEDE, GORIS and DE BRANDT, 1969). There is thus a system of control of inactivation and activation of phosphorylase a phosphatase analogous to that for phosphorylase b kinase. These two systems, dependent on activation and inhibition by nucleotides, are linked in such a manner that when one system is potentiated the other is inhibited and vice versa in a reciprocal manner.

After many inconclusive studies, a glycogen particle has finally been characterised. A protein-glycogen complex was isolated from rabbit muscle, which contained glycogen granules and fragments of the sarcoplasmic reticulum (SR) to which were attached phosphorylase, phosphorylase kinase, phosphorylase phosphatase and other enzymes (MEYER, HEILMEYER, HASCHKE and FISCHER,

1970). It was established that phosphorylase phosphatase included within this protein complex undergoes a reversible inhibition when phosphorylase is activated. This inhibition requires free Ca^{++} in addition to Mg-ATP, the same conditions that promote phosphorylase activation. Inhibition is only observed in the intact protein-glycogen complex. It was also established that the inhibition was not caused by AMP or IMP generated from the breakdown of ATP. AMP and IMP are known to inhibit phosphorylase a phosphatase by binding to the substrate, phosphorylase a (NOLAN, NOVOA, KREBS and FISCHER, 1964). Dissociation of the protein-glycogen complex by dilution leads to Ca^{++}-stabilization of phosphorylase a phosphatase with respect to Ca^{++} and a reappearance of its sensitivity towards AMP or IMP.

There is no evidence that changes occur in the enzyme during the process of inhibition and it is thought that inhibition results from interaction of phosphorylase a phosphatase with some of the other components of the phosphorylase system.

The activity of phosphorylase a phosphatase is amenable to alteration in a manner which suggests that the enzyme could play an active role in the regulation of glycogen phosphorylase (HASCHKE, HEILMEYER, MEYER and KREBS, 1970).

D. PHOSPHOFRUCTOKINASE (PFK)

PFK is the enzyme which catalyzes the reaction:

$$\text{FRUCTOSE-6-PHOSPHATE} + \text{ATP} \xrightarrow{Mg^{++}} \text{FRUCTOSE-1,6-DIPHOSPHATE} + \text{ADP}$$

The reaction is not significantly reversible. The reverse reaction is catalyzed by another enzyme, fructose-1,6-diphosphatase. PFK is now well established as a rate-limiting enzyme in glycogenolysis. The PFK step can either be activated or inhibited.

Rate limiting steps in the glycolytic pathway can be traced by applying the crossover theorem (CHANCE, HOLMES, HIGGENS and CONNELLY, 1958). Evidence for enzyme activation is based

on a fall in the concentration of the substrate and a rise in that of the product of the reaction. Inhibition is based on an accumulation of the substrate and a decline in the concentration of the products of the reaction.

In the PFK system, a crossover is observed between fructose-6-phosphate and fructose-1,6-diphosphate. An increased forward flux, lowering of fructose-6-phosphate and a rise in fructose-1,6-diphosphate due to activation of PFK is witnessed, and a decreased backward flux, concomitant increase in fructose-6-phosphate and fall-off in fructose-1,6-diphosphate is ascribable to inhibition of PFK.

According to these criteria, an increased flux due to PFK activation has been observed during (i) carbon monoxide-induced anoxia in rat heart (WILLIAMSON, 1966) (ii) alkalosis in the isolated rat heart (SCHEUER and BERRY, 1967) (iii) ischaemia of mouse brain (LOWRY, PASSONNEAU, HASSELBERGER and SCHULZ, 1964) (iv) muscular contraction in frog skeletal muscle (ÖZAND and NARAHARA, 1964) (v) insulin administration to frog skeletal muscle (ÖZAND and NARAHARA, 1964) (vi) exposure of mice to

the convulsant, Indoklan [(bis (2,2,2-trifluoroethyl ether)] (SACKTOR, WILSON and TIEKERT, 1966) (vii) in the blowfly, Phormia regina, during flight (SACKTOR and WORMSER-SHAVIT, 1966) (viii) during perfusion of rat heart with glucose, insulin and iodoacetamide (WILLIAMSON, 1967) and (ix) during electrical discharge of the electric organ of Electrophorus electricus (WILLIAMSON, CHEUNG, COLES and HERCZEG, 1967).

A decreased forward flux has been observed (i) during perfusion of rat heart with glucose, insulin and fluoroacetate (WILLIAMSON, 1967), (ii) with acetate and pyruvate (WILLIAMSON, 1965), or with fatty acids (GARLAND, RANDLE and NEWSHOLME, 1963; PARMEGGIANI and BOWMAN, 1963). All observations are consistent with activation of phosphofructokinase in conditions of enhanced glycolytic flux and vice versa.

PFK has been isolated and purified from guinea pig heart (MANSOUR, 1963), from rabbit muscle (PARMEGGIANI and KREBS, 1965; LING, MARCUS and LARDY, 1965) and from sheep heart (MANSOUR, WAKID and SPROUSE, 1966). Storage of the crystallized rabbit muscle PFK in the

presence of ATP prevents the enzyme from aggregating (PARMEGGIANI, LUFT, LOVE and KREBS, 1966). 2M NaCl caused dissociation of sheep heart PFK, accompanied by a decline in its catalytic activity (MANSOUR, WAKID and SPROUSE, 1966).

Incubation of rabbit skeletal muscle PFK with 5,-5'-dithiobis(2-nitrobenzoic acid) (DTNB) at pH 8,0 leads to a reaction with seven to eight cysteinyl residues per 10^5g of enzyme. At pH 7,0 approximately three thiol groups react rapidly with DTNB whilst the remaining four to five react slowly. By reaction of the first three -SH groups with DTNB, 90 - 95% of the enzyme activity is lost. In the presence of fructose-6-phosphate or adenine nucleotides, however, only one cysteinyl residue is highly reactive and there is, consequently, only a slight depression of the enzyme activity. Even after further reaction with the other four to five less reactive cysteinyl residues, enzyme activity is still retained. Glucose-6-phosphate, GMP and IMP do not possess the protective capacity of fructose-6-phosphate and other

adenine nucleotides (KEMP and FOREST, 1968). It transpires that PFK is a sulphydryl enzyme but that the reactive -SH groups can be protected by cofactors or substrate. A Mg-ATP complex also guards the rapidly reactive -SH groups of rabbit skeletal muscle PFK against attack by DTNB, through interaction with the inhibitory site on the enzyme (KEMP, 1969).

ATP and citrate are proven potent inhibitors of PFK, whilst ADP, AMP, cyclic AMP, inorganic phosphate, fructose-6-phosphate, fructose-1,6-diphosphate, ammonia and glucose-1,6-diphosphate are activators.

PFK from different tissues has exhibited many similarities in behaviour towards these inhibitors and activators.

Cyclic AMP (10^{-5}M) stimulates PFK activity in homogenates of guinea pig heart (MANSOUR, CLAGUE and BEERNINK, 1962). Extent of activation is inversely proportional to fructose-6-phosphate concentration. Cyclic AMP also partially protects PFK from inhibition by an excess of ATP. AMP and inorganic phosphate also activate the enzyme. ATP in concentrations greater than optimal inhibits PFK.

Incubation of the intact liver fluke with serotonin results in an increase of glycolysis, due to the increase of PFK activity (MANSOUR, 1962). Cyclic AMP (10^{-5}M) in this organism provokes a striking response in PFK. The stimulatory process requires the presence of both ATP and Mg^{++} ions. Activation due to cyclic AMP is inversely proportional to the concentration of fructose-6-phosphate. ATP in concentrations higher than optimal inhibits PFK (MANSOUR and MANSOUR, 1962), and cyclic AMP is protective against ATP under these conditions. This is an interesting example of enzyme inhibition by a necessary cofactor or substrate.

PFK can be isolated from rabbit skeletal muscle in two different forms, one containing nucleic acid and one that is free of nucleic acid. These two isoenzymes have different affinities for citrate, AMP, inorganic phosphate and fructose-1,6-diphosphate (HOFER and PETTE, 1966).

Rabbit muscle PFK is inhibited by ATP and stimulated by inorganic phosphate, AMP, ADP, fructose-6-phosphate and fructose-1,6-diphosphate.

Apparently, there are two sites of attachment for ATP, a primary active site and a second inhibitory site. The stimulators or activators can thus compete with ATP for the second site (PASSONNEAU and LOWRY, 1962).

The mode of binding of metabolites by rabbit skeletal muscle PFK has been studied by the gel filtration technique. ADP, cyclic AMP and AMP were bound competitively to PFK with dissociation constants of 0,5, 0,6 and 1,8 µM respectively. The dissociation constant of cyclic AMP bound to enzyme is lowered by the presence of fructose-6-phosphate and fructose-1,6-diphosphate.

Binding of PFK to fructose-6-phosphate, AMP, ADP and cyclic AMP indicated a molar binding unit of 90 000 daltons for these compounds, but three moles of ATP were bound by this quantity of enzyme. ATP inhibits the binding of fructose-6-phosphate and this inhibition was relieved by inorganic phosphate. Citrate enhances the affinity of PFK for ATP and weakens the affinity of the enzyme for AMP and fructose-6-phosphate (KEMP and KREBS, 1967).

Isoenzymes of PFK in E. coli (ATKINSON and WALTON, 1965), in sheep brain and in rat liver (PASSONNEAU and LOWRY, 1963) exhibit essentially similar patterns of inhibition and stimulation. Yeast PFK, however, does react somewhat differently in that incubation of the enzyme with trypsin renders it insensitive to ATP (SALAS, SALAS and SOLS, 1968). Many other phosphorylated intermediates have been studied, including creatine phosphate and 3-phosphoglycerate and the latter has been recognised to be the most potent inhibitor of PFK isolated from rabbit brain (KRZANOWSKI and MATSCHINSKY, 1969).

Partially purified preparations of PFK from guinea pig heart, when incubated at pH 5,8, lose activity reversibly. The inactive enzyme can be revivified in an alkaline pH. Reactivation is expedited by the following nucleotides and hexose phosphates; ATP, ADP, AMP, fructose-6-phosphate and fructose-1,6-diphosphate. A mixture of a nucleotide with a hexose phosphate is much more effective in reactivation of PFK than either nucleotide or hexose phosphate alone (MANSOUR, 1965). The

enzymic activity of PFK of the skeletal muscle from frog and mouse is extremely sensitive to small changes in pH in the physiological range. A low pH weakens the affinity of the enzyme for fructose-6-phosphate. Shifts in intracellular pH may be important in the regulation of PFK activity (TRIVEDI and DANFORTH, 1966). A fall in intracellular pH is likely to inhibit PFK and glycolysis would tend, therefore, to be self-limiting. On the other hand, its pH dependence could be invoked to explain the tendency towards lactacidosis observed during alkalosis in man (GEVERS and DOWDLE, 1963).

E. PYRUVATE KINASE (PK)

Pyruvate kinase (PK) catalyzes the reaction

$$\text{PHOSPHOENOLPYRUVATE} + \text{ADP} \xrightarrow[K^+;\ Mg^{++}]{\text{PYRUVATE KINASE}} \text{PYRUVATE} + \text{ATP}$$

(LARDY and ZIEGLER, 1945).

Rabbit muscle PK has a molecular weight of 237 000 daltons. It is a tetramer, containing four polypeptide chains, each with a molecular weight of 57 200. PK is regarded as consisting of two identical catalytic units or protomers, each with a molecular weight of 115 000, each protomer in turn comprising two polypeptide chains, each with a molecular weight of 57 000. Each protomer has a single site for Mn^{++} and phosphoenolpyruvate. There is also speculation that the two polypeptide chains within a protomer might not be identical, despite their similarity in molecular weight. Treatment with urea revealed intermediates with a sedimentation rate of 7,3S in 1,5M urea and particles of 3,6S in 3M urea (STEINMETZ and DEAL, 1966).

Potassium acts as a potent activator of rabbit muscle PK, and both Rb^{++} and NH_4^+ can replace K^+ in this role. Although Na^+ is a weak activator, Li^+ has no or little adjuvant capacity. Both Na^+ and Li^+ were found to oppose the action of K^+ (KACHMAR and BOYER, 1953). The presence of ADP is also obligatory for the PK reaction (HARRISON, BOYER and FALCONE, 1955). Mg^{++} has been found to be an activator of PK

of mammalian erythrocytes, and can be partially replaced by Mn^{++} or Co^{++} (SOLVONUK and COLLIER, 1955).

PK, purified from mammalian erythrocytes, requires free thiol functions for its activity. Complete inhibition of PK is observed on addition of 10^{-4}M p-CMB and this inhibition can be partially revoked by glutathione (SOLVONUK and COLLIER, 1955). In rat muscle, inorganic phosphate and Na^{+}, in relative high concentrations, inhibit the transfer of phosphate from phosphoenolpyruvate to ADP (BOYER, LARDY and PHILLIPS, 1943).

Low concentrations of Ca^{++} in rabbit muscle prevent the activation of PK by K^{+}. Approximately 50% inhibition was observed in the presence of 0,05M K^{+} and 0,002M Ca^{++}. Raising the concentration of K^{+},however, partly reversed the inhibition by Ca^{++} (KACHMAR and BOYER, 1953). The negatory effect of Ca^{++} is due to prohibition of the transfer of phosphate from phosphoenolpyruvate to ADP (BOYER, LARDY and PHILLIPS, 1943), and is competitive with Mn^{++} and Mg^{++} (MILDVAN and COHEN, 1965).

ATP also inhibits PK, and this inhibition is competitive with phosphoenolpyruvate (BOYER, 1969), from the fact that their transferable phosphate groups bind to the same site on PK (REYNARD, HASS, JACOBSEN and BOYER, 1961).

Partially purified erythrocyte PK can be stabilized by Mg^{++}, 2-mercaptoethanol and inorganic phosphate. Phosphoenolpyruvate was found to be much more effective for this than ADP or inorganic phosphate. Ethylenediaminetetraacetic acid (EDTA) impaired this stabilizing effect, maybe due to chelation of the Mg^{++} ions. This isoenzyme was much more stable below pH 7,4 than above, and the optimum pH lay between pH 6,8 and pH 7,1 (IBSEN, SCHILLER and VENN-WATSON, 1968).

Rat epididymal adipose PK exists in two interconvertible forms, designated as PK-A, which is activated by fructose-1,6-diphosphate, and PK-B which is insensitive to fructose-1,6-diphosphate (POGSON, 1968 (a) and (b)). PK-A can be converted to PK-B by incubation with low concentrations of fructose-1,6-diphosphate and this process is reversible by incubation with EDTA, ATP and citrate which compete with

fructose-1,6-diphosphate. During active glycolysis, this enzyme is normally present in the PK-B form. Incubation of epididymal fat pads with glucose and insulin provides evidence that the equilibrium under surveillance of PK in this tissue is far displaced towards pyruvate, and PK serves as a control point during glycolysis.

There are two types of PK present in mammalian tissues, namely type L and type M. The M type is present in muscle, brain, heart, liver and kidney, but the L type is present only in kidney and liver (TANAKA, HARANO, MORIMURA and MORI, 1965). Crystalline L type from rat liver can be activated by fructose-1,6-diphosphate or phosphoenolpyruvate, and inhibition by ATP was markedly alleviated by the presence of fructose-1,6-diphosphate. The M type on the other hand did not respond significantly to the presence of fructose-1,6-diphosphate. The reaction rate of PK in rat liver can therefore be controlled with fine precision, in vivo, by the presence, in varying concentrations, of ATP, phosphoenolpyruvate and fructose-1,6-diphosphate (TANAKA, SUE and MORIMURA, 1967). The L type

PK of mice is strongly inhibited by Cu^{++}, but this inhibition was also reversible by the addition of fructose-1,6-diphophate. The M isoenzyme was only slightly or not at all affected by Cu^{++} (PASSERON, DE ASUA and CARMINATTI, 1967).

Studies with rat liver PK in vitro also indicate a maximal activation of the enzyme when it is saturated with certain concentrations of NADH. However, if the NADH concentration is further raised (> 0.3 µmoles/ml reaction mixture), PK is gradually inactivated and activity approaches zero. If this interaction operates in vivo, it could provide a mechanism for switching off PK when lactate is converted into pyruvate during gluconeogenesis (WEBER, SINGHAL, STAMM AND SRIVASTAVA, 1965). The rate of change of lactate to pyruvate within the liver, part of the Cori cycle of lactate, will, of course, be of supreme importance in conditions giving rise to lactate acidosis. The theme of this thesis, Malignant Hyperthermia, provides a situation in which generation of NADH by the lactate dehydrogenase system will be amplified many

fold as compared with the resting state. Studies with rat liver PK have also proven that triamcinolone, a steroid and potent stimulator of gluconeogenesis, is without effect on hepatic PK. PK activity in diabetes and the response of the enzyme to insulin have also been studied. When alloxan-diabetes was induced by intraperitoneal injection, it was noted that activity of PK fell to 60% at 24 hours and to 30-40% at 144 hours after induction of diabetes (WEBER, STAMM and FISHER, 1965). At this stage, insulin had almost disappeared from the alloxan-treated rat. To investigate the action of insulin on these diabetic rats, insulin was injected, and it was observed that PK activity was then restored to normal, due to synthesis of new enzyme induced by the action of insulin.

Dietary conditions also play a role in the regulation of activity of the PK enzyme (KREBS and EGGLESTON, 1965). On changing from a standard diet to a low carbohydrate diet, enzyme activity declined to about one third, and on a high carbohydrate diet, the

activity rose to more than three fold. When the diet was low in carbohydrates or during starvation, gluconeogenesis occurs on a major scale, and PK activity is correspondingly low, and the conversion of phosphoenolpyruvate to pyruvate is retarded or abated completely. On the other hand, when the diet is rich in carbohydrates, PK activity is greatly raised and this promotes the conversion of phospho-enolpyruvate to pyruvate, which, in turn, is decarboxylated to acetyl CoA, which can either be oxidized in the citric acid cycle or be utilized for synthesis of fatty acids. The activity of PK reaches a maximum, on this regime, only after a few days have elapsed, from which it is inferred that the augmented activity stems from a more rapid rate of enzyme synthesis. This alteration in PK activity provides an important regulatory device for changing over from storage of glycogen to fat storage.

PART II

M E T H O D O L O G Y

I. EXPERIMENTAL MODEL

Pigs known to be susceptible to halothane, on the grounds either of previous exposure or of an observed elevated plasma creatine phosphokinase activity, were anaesthetised with pentothal intravenously and intubated. Anaesthesia was maintained with intravenous pentothal and nitrous oxide/oxygen (80/20 v/v). Adductor magnus, a muscle in the groin, was exposed and longitudinal strips were carefully freed from the underlying tissue and string ligatures were placed loosely round their proximal and distal ends.

At time 0, halothane 3% v/v was introduced into the inhalation circuit. None of the pigs in this series reacted to halothane alone. Between 10 and 15 minutes later, 2 mg succinyl choline was administered intravenously. All animals experienced a rapid rise in body temperature to 42 or 43°C within 20 minutes. Pigs 22B and 139 developed rigor which was sustained until death 30-40 minutes later. This rigor persisted unchanged into post-mortem. Pig 137 was anomalous in that although hyperpyrexia

ensued, there was no rigor and lactacidosis was less severe (see Results).

II. ENZYMES

Fructose-1,6-diphosphate aldolase was obtained from Boehringer and Soehne GmbH, Mannheim, Germany. Creatine phosphokinase, enolase, glucose-6-phosphate dehydrogenase, ∝-glycerophosphate dehydrogenase, myokinase, malic dehydrogenase, phosphoglucose isomerase, phosphoglucomutase, phosphoglycerate mutase, pyruvate kinase and triosephosphate isomerase came from Sigma Chemical Company, St. Louis, U.S.A. Hexokinase and lactic dehydrogenase were products from Seravac Laboratories, Cape Town.

III. PREPARATION OF TISSUE EXTRACTS

PCA extracts were prepared according to WILLIAMSON, 1965. Samples of skeletal muscle were frozen within 5 to 8 seconds after removal by immersing immediately into liquid nitrogen. The frozen tissue was powdered in a stainless steel percussion mortar, which had been cooled in liquid nitrogen. The powdered tissue was transferred into a preweighed McCartney bottle containing cold PCA 0,6N which was kept in ice. The McCartney bottle was weighed again to obtain the weight of the tissue. For each gram of tissue, 4 volumes of PCA (0,6N) was added. The tissue was homogenized for a few seconds in the cold ($4^{\circ}C$) with an Ultra Turrax homogenizer. The mixture was transferred into a centrifuge tube and centrifuged at 15 000 x g for 15 minutes in a refrigerated Sorvall RC-2B centrifuge. The volume of supernatant was measured and the pH adjusted to 6,0 by addition of K_2CO_3 (3,0N), the volume of K_2CO_3 needed being recorded. The potassium perchlorate precipitate was removed by centrifugation at 15 000 x g for 15 minutes in a refrigerated Sorvall RC-2B

centrifuge. Extracts were kept frozen if not used immediately. The least stable glycolytic intermediates such as pyruvate, phosphoenolpyruvate, 2-phosphoglycerate, 3-phosphoglycerate were analyzed on the day of extraction. The other glycolytic intermediates and cofactors were measured over a period of days.

IV. QUANTITATION OF GLYCOLYTIC INTERMEDIATES BY MEANS OF ENZYMATIC NUCLEOTIDE-LINKED REACTIONS.

A. PRINCIPLES OF METHODS

Measurements were based on the principle of (i) a decrease in O.D. at 340 nm due to the oxidation of NADH to NAD or (ii) a rise in O.D. at 340 nm due to the reduction of NADP to NADPH or NAD to NADH. The change in O.D. was measured spectrophotometrically in a Zeiss PMQII spectrophotometer. Because of their low concentrations, dihydroxyacetone phosphate and glyceraldehyde-3-phosphate were determined

fluorometrically. Fluorometric measurements were made in a Hitachi Perkin-Elmer Fluorescence Spectrophotometer MPF-2A. Excitation was at 340 nm and emission was set at 420 nm.

The substrates, fructose-1,6-diphosphate, 2-phosphoglyceric acid, 3-phosphoglyceric acid, phosphoenolpyruvate, adenosine-5'-monophosphate and adenosine-5'-diphosphate were quantitated by following the oxidation of NADH.

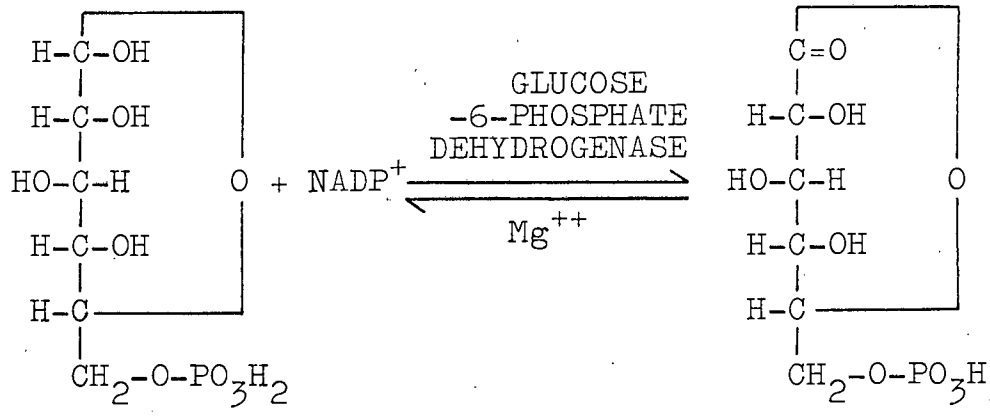
Reduction of NAD^+ was used to measure lactate, α-glycerophosphate, malate, dihydroxyacetone phosphate and glyceraldehyde-3-phosphate.

Reduction of NADP, coupled with glucose-6-phosphate dehydrogenase, provided the basis for determinations of glucose-1-phosphate, glucose-6-phosphate, creatine phosphate and adenosine-5'-triphosphate.

DETERMINATION OF GLUCOSE-1-PHOSPHATE,
GLUCOSE-6-PHOSPHATE and FRUCTOSE-6-PHOSPHATE

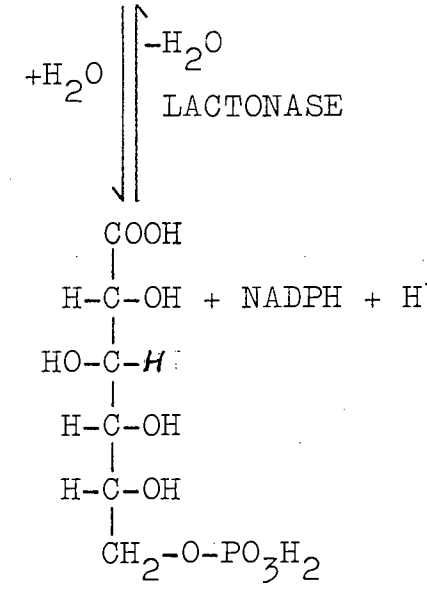
BERGMEYER and KLOTESCH, 1965; KLOTESCH and BERGMEYER, 1965.

Principle:

(1) 

GLUCOSE-6-PHOSPHATE

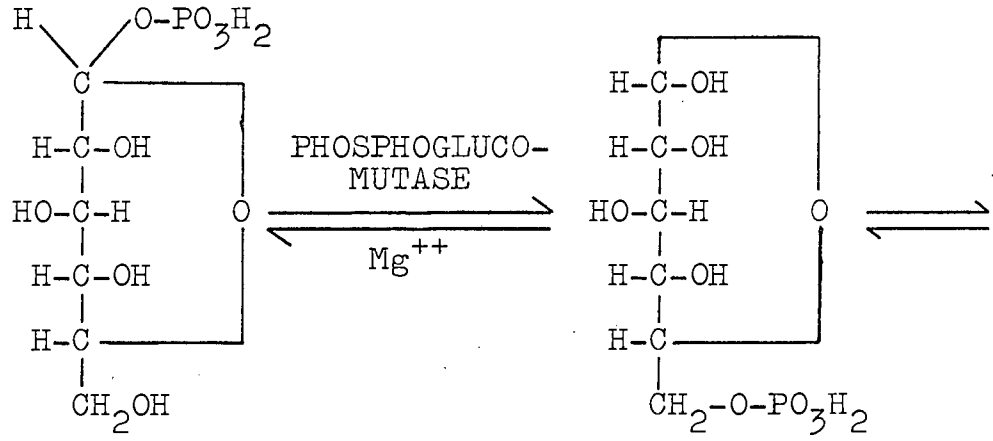
6-PHOSPHOGLUCONO-δ-LACTONE



6-PHOSPHOGLUCONIC ACID

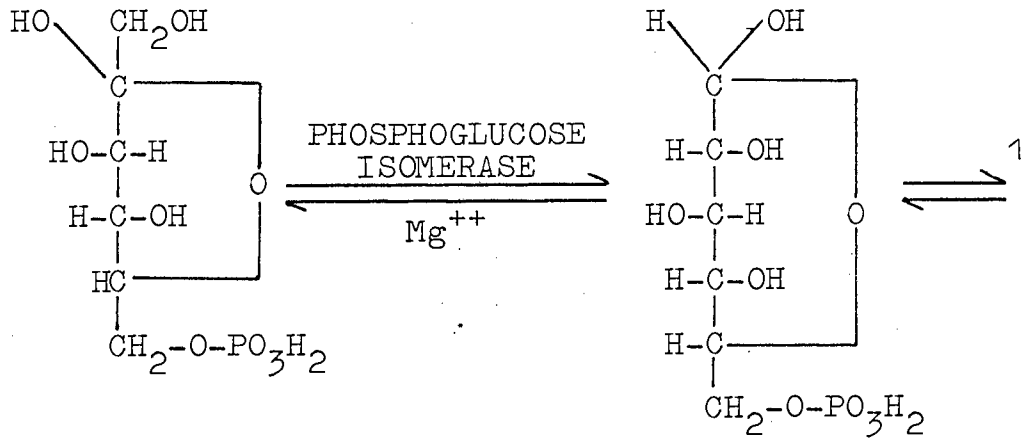
Glucose-6-phosphate is oxidized by NADP and glucose-6-phosphate dehydrogenase to 6-phosphogluconic acid. The equilibrium of this reaction lies far to the right, that is, to the formation of 6-phosphogluconic acid and NADPH.

(2)



GLUCOSE-1-PHOSPHATE GLUCOSE-6-PHOSPHATE

Glucose-1-phosphate is converted to glucose-6-phosphate under the influence of the enzyme phosphoglucomutase in the presence of Mg^{++} ions. The glucose-6-phosphate is oxidized according to equation 1, page 59.

(3) 

FRUCTOSE-6-PHOSPHATE GLUCOSE-6-PHOSPHATE

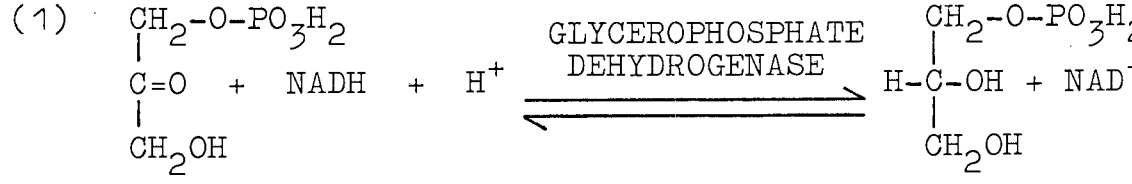
Fructose-6-phosphate is converted to glucose-6-phosphate with the aid of phosphoglucose isomerase, and glucose-6-phosphate again processed according to equation 1, page 59.

DETERMINATION OF FRUCTOSE-1,6-DIPHOSPHATE, DIHYDROXYACETONE PHOSPHATE AND GLYCERALDEHYDE-3-PHOSPHATE.

(BÜCHER AND HOHORST, 1965)

Principle:

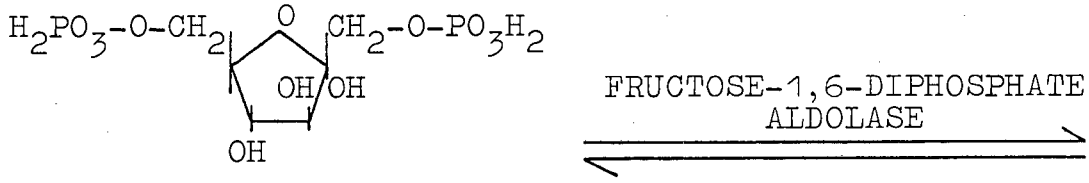
(1)



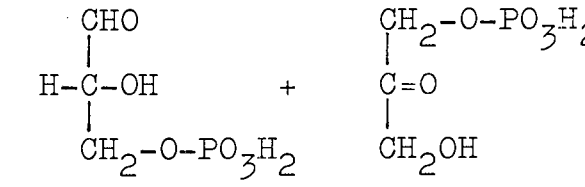
DIHYDROXYACETONE PHOSPHATE

α-GLYCEROPHOSPHATE

(2)



FRUCTOSE-1,6-DIPHOSPHATE

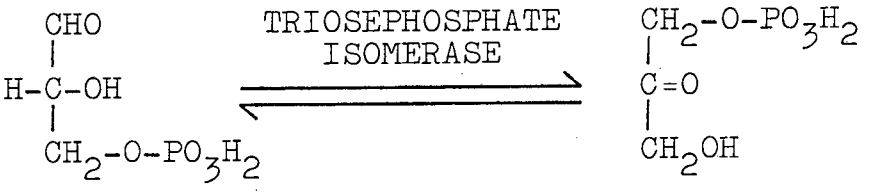


GLYCERALDEHYDE-3-PHOSPHATE

DIHYDROXY-ACETONE PHOSPHATE

Fructose-1,6-diphosphate aldolase cleaves fructose-1,6-diphosphate between carbon 3 and 4 to give two triose phosphates, glyceraldehyde-3-phosphate and dihydroxy-acetone phosphate.

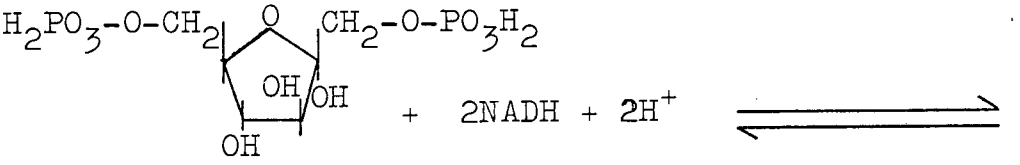
(3)



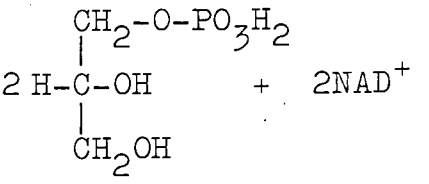
GLYCERALDEHYDE-3-PHOSPHATE

DIHYDROXYACETONE PHOSPHATE

(4)



FRUCTOSE 1,6-DIPHOSPHATE



∝-GLYCEROPHOSPHATE

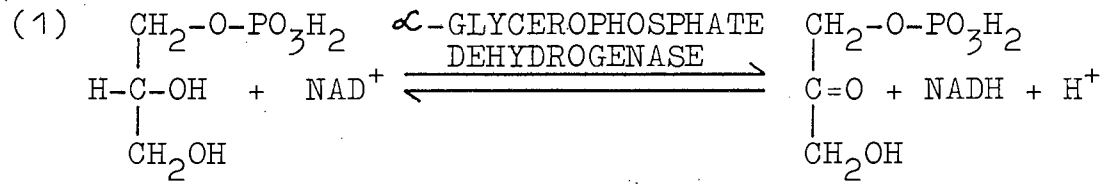
For each molecule of fructose 1,6-diphosphate, 2 molecules of NADH are oxidized to $2NAD^+$.

DETERMINATION OF α-GLYCEROPHOSPHATE

(HOHORST, 1965 (c))

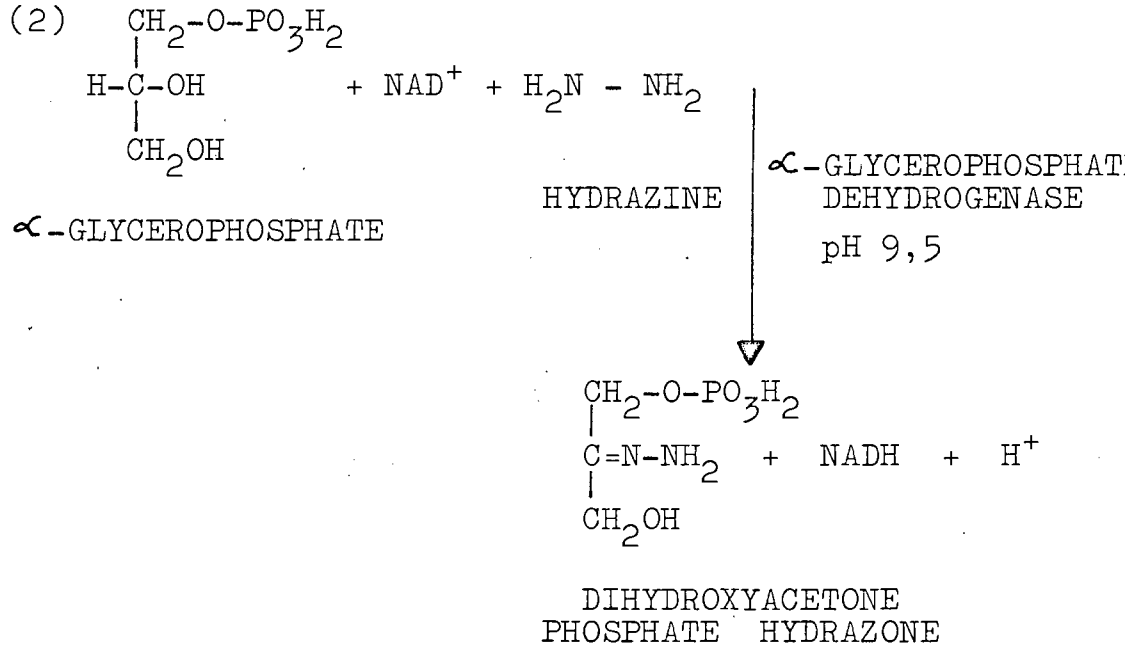
Principle:

(1)



α-GLYCEROPHOSPHATE — DIHYDROXYACETONE PHOSPHATE

(2)



α-GLYCEROPHOSPHATE

DIHYDROXYACETONE PHOSPHATE HYDRAZONE

The equilibrium of the above reactions lies far to the left, in the direction of the formation of α-glycerophosphate. The reaction product,

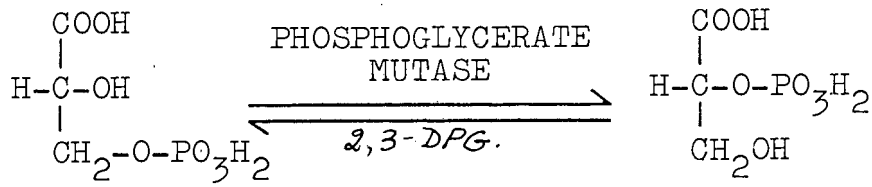
dihydroxyacetone phosphate, must therefore be removed continuously for the reaction to proceed. Protons formed are bound by an alkaline medium, and the reaction product, dihydroxyacetone phosphate, is trapped as dihydroxyacetone phosphate hydrazone.

DETERMINATION OF 3-PHOSPHOGLYCERATE, 2-PHOSPHOGLYCERATE, PHOSPHOENOLPYRUVATE AND PYRUVATE.

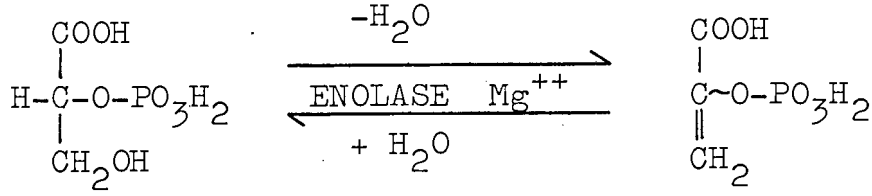
(CZOK and ECKERT, 1965)

Principle:

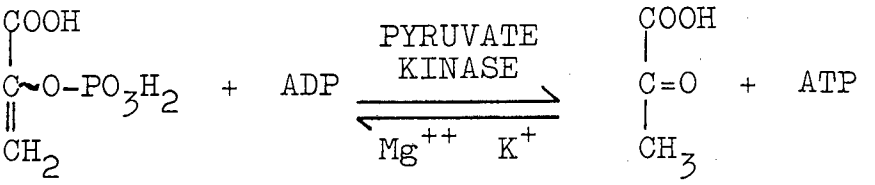
The substrates 3-phosphoglycerate, 2-phosphoglycerate, phosphoenolpyruvate and pyruvate were determined by the following reactions:

(1) 

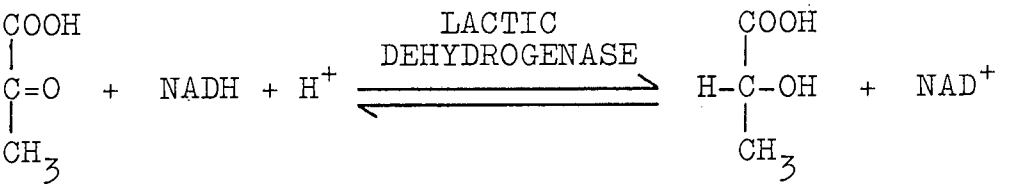
3-PHOSPHOGLYCERATE 2-PHOSPHOGLYCERATE

(2) 

2-PHOSPHOGLYCERATE PHOSPHOENOLPYRUVATE

(3) 

PHOSPHOENOLPYRUVATE PYRUVATE

(4) 

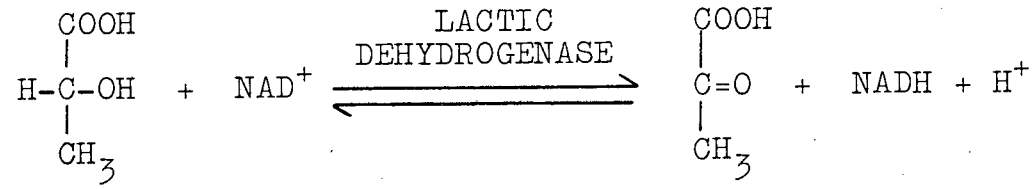
PYRUVATE LACTATE

3-phosphoglycerate can be determined by this method only if the assay mixture contains less than 10^{-3}M inorganic phosphate. The inhibitory effect of phosphate can be diminished if the same volume of $MnSO_4$ is added instead of $MgSO_4$. Phosphate does not interfere with determination of 2-phosphoglycerate, phosphoenolpyruvate and pyruvate.

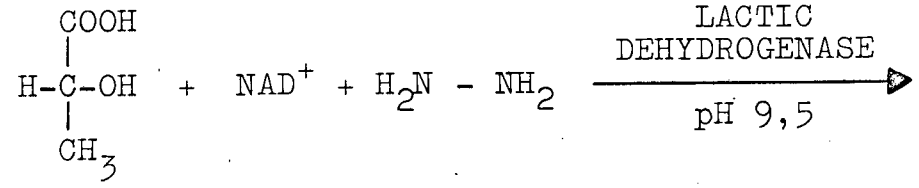
DETERMINATION OF LACTATE

(HOHORST, 1965 (a))

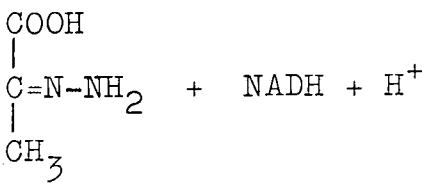
Principle:

(1) 

LACTATE PYRUVATE

(2) 

LACTATE HYDRAZINE



PYRUVATE HYDRAZONE

The equilibrium of reaction lies far to the left, that is, towards lactate. The reaction product pyruvate, must therefore, be removed from

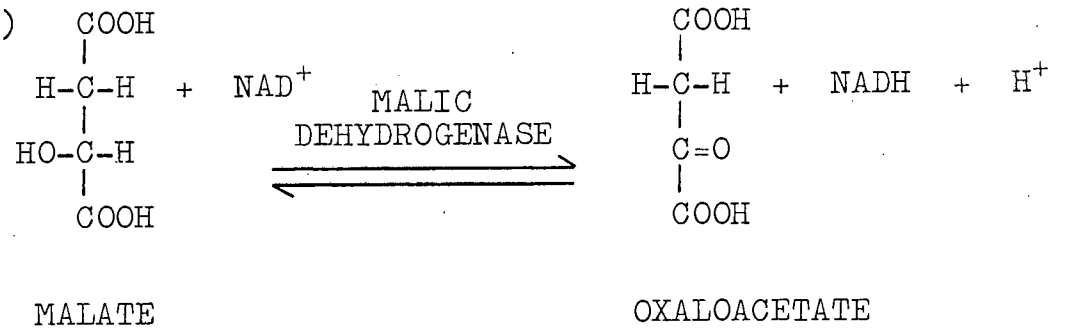
the reaction mixture, as fast as it is formed, to allow continuous oxidation of lactate. Protons are neutralized by having an alkaline medium, pH 9,5, and pyruvate is trapped as pyruvate hydrazone.

DETERMINATION OF MALATE

(HOHORST, 1965 (b))

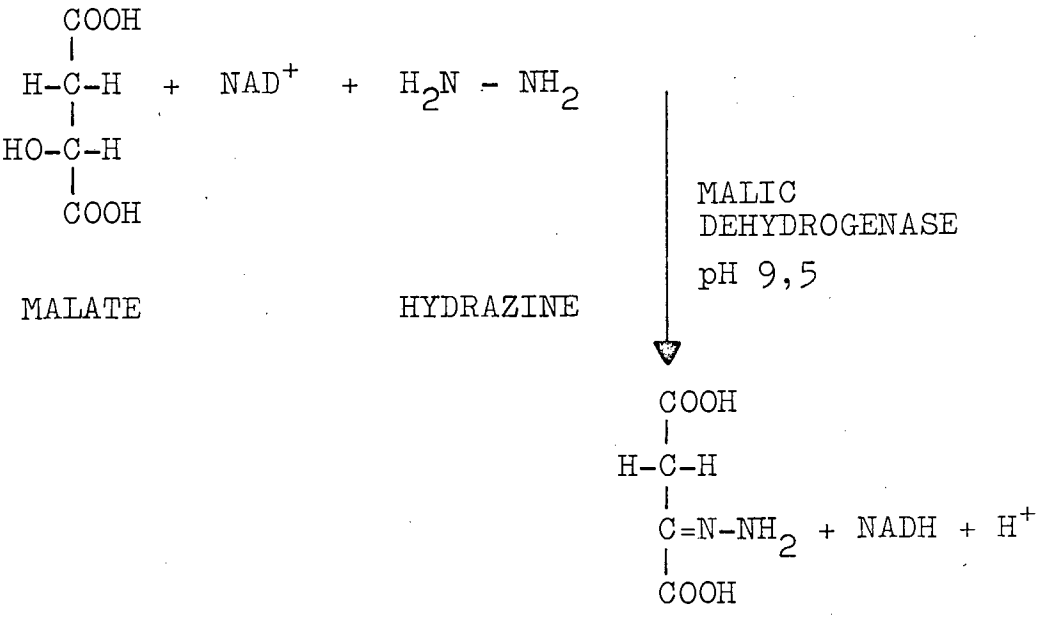
Principle:

(1)



MALATE OXALOACETATE

(2)



MALATE HYDRAZINE

OXALOACETATE HYDRAZONE

The equilibrium of the above reaction (1) also lies far to the left, that is, in the direction of the formation of malate. The reaction product, oxaloacetate, must therefore be

removed for the reaction to proceed. The protons formed are removed into the alkaline medium, and the reaction product, oxaloacetate, is trapped as oxaloacetate hydrazone.

DETERMINATION OF ADENOSINE-5'-MONOPHOSPHATE AND ADENOSINE-5'-DIPHOSPHATE

(ADAM, 1965)

Principle:

(1) $AMP + ATP \underset{Mg^{++}}{\overset{MYOKINASE}{\rightleftharpoons}} 2\ ADP$

(2) $2\ C(COOH)(=CH_2)(-O-PO_3H_2) + 2\ ADP \underset{Mg^{++}\quad K^{+}}{\overset{PYRUVATE\ KINASE}{\rightleftharpoons}} 2\ C(COOH)(=O)(CH_3) + 2\ ATP$

PHOSPHOENOLPYRUVATE — PYRUVATE

(3) $2\ C(COOH)(=O)(CH_3) + 2\ NADH + 2\ H^{+} \underset{}{\overset{LACTIC\ DEHYDROGENASE}{\rightleftharpoons}} 2\ H-C(COOH)(OH)(CH_3) + 2\ NAD^{+}$

PYRUVATE — LACTATE

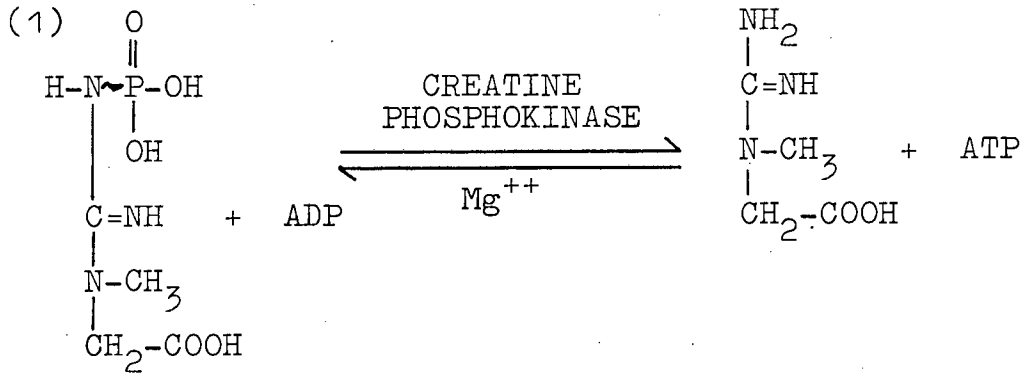
AMP is phosphorylated in the presence of ATP and myokinase to form two molecules of ADP. Phosphoenolpyruvate possesses a high energy phosphate bond which permits it to react with ADP to form ATP and pyruvate. The reaction is catalyzed by the enzyme pyruvate kinase which requires Mg^{++} and K^{+} ions.

DETERMINATION OF CREATINE PHOSPHATE AND ADENOSINE-5'-TRIPHOSPHATE

(LAMPRECHT and STEIN, 1965)

Principle:

(1)



CREATINE PHOSPHATE CREATINE

The enzyme creatine phosphokinase catalyzes the transfer of the high energy phosphate of creatine phosphate to ADP, to form creatine and ATP. ATP in turn phosphorylates glucose in the presence of hexokinase, to form glucose-6-phosphate, with the regeneration of ADP. Glucose-6-phosphate dehydrogenase catalyzes the oxidation of glucose-6-phosphate by its cofactor $NADP^+$ to form 6-phosphogluconic acid.

(2)

$$\text{GLUCOSE} + \text{ATP} \xrightleftharpoons[Mg^{++}]{\text{HEXOKINASE}} \text{GLUCOSE-6-PHOSPHATE} + \text{ADP}$$

(3)

$$\text{GLUCOSE-6-PHOSPHATE} + \text{NADP}^{+} \xrightarrow[\text{DEHYDROGENASE}]{\text{GLUCOSE-6-PHOSPHATE}} \text{6-PHOSPHOGLUCONO-}\delta\text{-LACTONE}$$

$$\text{6-PHOSPHOGLUCONO-}\delta\text{-LACTONE} \xrightleftharpoons{\text{LACTONASE}} \text{6-PHOSPHOGLUCONIC ACID} + \text{NADPH} + \text{H}^{+}$$

(4) <u>The overall reaction is</u>:

$$\text{CREATINE PHOSPHATE} + \text{GLUCOSE} + \text{NADP}^{+} \rightleftharpoons \text{CREATINE} + \text{6-PHOSPHOGLUCONIC ACID} + \text{NADPH} + \text{H}^{+}$$

For each molecule of creatine phosphate decomposed, one molecule of NADPH is formed.

Calculation of Results

The following formula was applied to calculate the concentration of compounds originally present in sample of muscle.

$$\frac{\Delta E \times V}{\varepsilon \times V_D \times d} \times \frac{\text{total volume of PCA extract}}{\text{weight of muscle sample taken}} = \mu\text{moles/g.}$$

where

ΔE = change in O.D. 340 nm

V = volume of the assay mixture (ml)

V_D = volume of the PCA extract in the assay mixture (ml)

d = light path of the cuvette (cm)

ε = millimolar extinction coefficient of NADH or NADPH ($/cm^2/$ μmole)

$\varepsilon^{\text{NADH(mM)}}_{340\text{ nm}} = 6,22$

DETERMINATION OF BLOOD LACTATE

(HOHORST, 1965 (a))

Equal volumes of blood and of PCA were mixed in a glass-stoppered tube and centrifuged for 20 minutes at 2 000 x g in a refrigerated Sorvall RC-2B centrifuge. The supernatant fluid was removed. Lactate was measured enzymatically in the PCA extract as for muscle, but omitting precipitation of perchlorate. Owing to the relatively high concentration of lactate in serum, and the small volume of extract which was added to the incubation mixture, the volume of PCA included was insufficient significantly to affect calculation of the activity of lactic dehydrogenase.

B. ASSAY PROCEDURES.

Operative conditions in the various assays, such as total volume, concentration of buffer and cofactors, pH are given in Table I (pp. 78, 79). Quantities of enzyme were chosen which would achieve equilibrium within 3 to 5 minutes. Reactions were initiated by addition of the appropriate volume of enzyme solution and followed at 1 minute intervals until complete. It was found convenient to record results and calculations on a duplicated sheet, a copy of which is reproduced in Fig. 5. In many instances, it was possible to determine substrates serially in the same reaction mixture by adding specific enzymes. The following were determined together

(a) Glucose-6-phosphate, glucose-1-phosphate and fructose-6-phosphate.

(b) Pyruvate, phosphoenolpyruvate, 2-phosphoglycerate and 3-phosphoglycerate.

(c) α-glycerophosphate and malate.

(d) ADP and AMP.

(e) ATP and creatine phosphate.

TABLE 1

Conditions for assay of glycolytic intermediates and cofactors

(Concentration in final reaction mixture)

Substrate	Vol. PCA extract ml.	Final Volume ml.	Light Path cm.	Buffer	Amount Buffer mM	Buffer pH	Cofactors	Amount Cofactors mM	Enzyme used	Enzyme Protein added mg.
Glucose-1-phosphate	0,5	3,70	2	Triethanolamine, 0,05 M	41	7,6	NADP $MgCl_2$	0,32 2,70	PGluM	0,04
Glucose-6-phosphate	0,5	3,70	2	Triethanolamine, 0,05 M	41	7,6	NADP $MgCl_2$	0,32 2,70	G6P-DH	$8,6 \times 10^{-4}$
Fructose-6-phosphate	0,5	3,70	2	Triethanolamine, 0,05 M	41	7,6	NADP $MgCl_2$	0,32 2,70	PGI	0,1
Fructose-1,6-diphosphate	0,2	2,05	1	Triethanolamine, 0,4 M	98	7,6	NADH	0,12	ALD	0,1
Dihydroxyacetone phosphate	0,2	2,05	1	Triethanolamine, 0,4 M	98	7,6	NADH	0,12	GDH	0,1
Glyceraldehyde-3-phosphate	0,2	2,05	1	Triethanolamine, 0,4 M	98	7,6	NADH	0,12	TIM	0,1
α-Glycerol phosphate	1,0	2,10	1	Hydrazine, 0,4 M Glycine, 1,0 M	667	9,5	NAD	2,38	GDH	0,1
3-Phosphoglycerate	1,5	2,25	1	Triethanolamine, 0,2 M	44	7,6	NADH KCl $MgSO_4$ ADP 2,3-diPG	0,09 67 7,10 0,22 0,11	PGM	0,1
2-Phosphoglycerate	1,5	2,52	1	Triethanolamine, 0,2 M	44	7,6	NADH KCl $MgSO_4$ ADP 2,3-diPG	0,09 67 7,10 0,22 0,11	ENOLASE	0,1

TABLE I (Continued)

Substrate	Vol. PCA extract ml.	Final Volume ml.	Light Path cm.	Buffer	Amount Buffer mM	Buffer pH	Cofactors	Amount Cofactors mM	Enzyme used	Enzyme Protein added mg.
Phosphoenolpyruvate	1,5	2,25	1	Triethanolamine, 0,2 M	44	7,6	NADH KCl $MgSO_4$ ADP 2,3-diPG	0,09 67 7,10 0,22 0,11	PK	0,2
Pyruvate	1,5	2,25	1	Triethanolamine, 0,2 M	44	7,6	NADH KCl $MgSO_4$ ADP 2,3-diPG	0,09 67 7,10 0,22 0,11	LDH	0,1
Lactate	0,05	2,10	1	Hydrazine, 0,4 M Glycine, 1,0 M	667	9,5	NAD	2,38	LDH	0,1
Malate	1,0	2,10	1	Hydrazine, 0,4 M Glycine, 1,0 M	667	9,5	NAD	2,38	MDH	0,025
Adenosine-5'-monophosphate	1,0	2,00	1	Triethanolamine, 0,05 M	215	7,55	NADH KCl $MgSO_4$ PEP ATP	0,69 1,74 0,82 0,92 0,16	MK	0,005
Adenosine-5'-diphosphate	1,0	2,00	1	Triethanolamine, 0,05 M	215	7,55	NADH KCl $MgSO_4$ PEP ATP	0,69 1,74 0,82 0,92 0,16	LDH PK	0,002 0,01
Adenosine-5'-triphosphate	0,1	5,24	2	Triethanolamine, 0,05 M	38	7,6	NADP $MgCl_2$ ADP Glucose	0,13 6,68 0,10 38	HK G6P-DH	0,05 $3,52 \times 10^{-3}$
Creatine phosphate	0,1	5,24	2	Triethanolamine, 0,05 M	38	7,6	NADP $MgCl_2$ ADP Glucose	0,13 6,68 0,10 38	CPK	0,05

FIG. 5.

DETERMINATION OF α-GLYCEROL-PHOSPHATE, MALATE. 24/2/1971

PIG 22 B

ecimen		-10'	-10'	+1'	+1'
- (Vol. of assay mixture)		2·1	2·1	2·1	2·1
= Vol. sample		1·0	1·0	1·0	1·0
= (light path in cm)		1	1	1	1
= Extinction coeff. of NAD.		6·22	6·22	6·22	6·22
BUFFER, SAMPLE, MIXT.		0·345	0·350	0·350	0·355
(0.01 ml α-Glyc-P	1'	0·548	0·549	0·556	0·558
	3'	0·590	0·592	0·608	0·610
	5'	0·602	0·602	0·622	0·630
	7'	0·612	0·612	0·632	0·642
	10'	0·614	0·616	0·640	0·650
	15'	0·616	0·618	0·642	0·654
$E_2 - E_1 = \Delta E_{\alpha\text{-GLYC-P}}$		0·271	0·268	0·292	0·299
(0.01 ml MALIC DEH.)	1'	0·650	0·644	0·668	0·680
	3'	0·650	0·648	0·674	0·684
	5'	0·654	0·652	0·680	0·690
	7'				
	10'	0·658	0·660	0·690	0·702
$E_3 - E_2 = \Delta E_{MALATE}$		0·042	0·042	0·048	0·048
[α-GLYCERO-P]	ΔE	0·264	0·261	0·285	0·292
µM/ML		0·089	0·088	0·096	0·099
µM/G		0·465	0·460	0·503	0·518
[MALATE]	ΔE	0·032	0·032	0·038	0·038
µM/ML		0·011	0·011	0·013	0·013
µM/G		0·058	0·058	0·068	0·068

CALCULATION : $\frac{E \times V}{\varepsilon \times V_d \times d}$ = µMOLES / ML

Such assays are feasible only with the use of pure enzymes. In every case, this was checked by failure of the enzyme to react with other than its own specific substrate. As an example, 0,01 ml ($8,6 \times 10^{-3}$ mg) of one preparation of glucose-6-phosphate dehydrogenase, in the presence of $NADP^+$, was found to react with both glucose-6-phosphate and fructose-6-phosphate (Fig. 6, p. 83). The inference was that the enzyme was contaminated with significant quantities of phosphoglucose isomerase. However, 0,001 ml ($8,6 \times 10^{-4}$ mg) of the same preparation catalyzed the dehydrogenation of glucose-6-phosphate at an acceptable rate. Reaction with fructose-6-phosphate was not appreciable when oxidation of glucose-6-phosphate had reached completion (Fig. 7, p. 84).

Change in optical density observed during typical assays are shown in Fig. 8 (p. 85). Possible contribution to change in optical density by the enzyme preparation itself was allowed for in a blank determination (no PCA extract) and by adding the same volume of enzyme solution at the end of the reaction period and noting any increase in optical

density. Where significant, a correction was applied to ΔE before final calculation of results.

Pure standard substrates were not available for checking recovery in every instance and the ε mM value of 6,22 was employed throughout. Assays were presumed to be satisfactory since, without exception, optical density changes of measurable magnitude were observed which became stable with time.

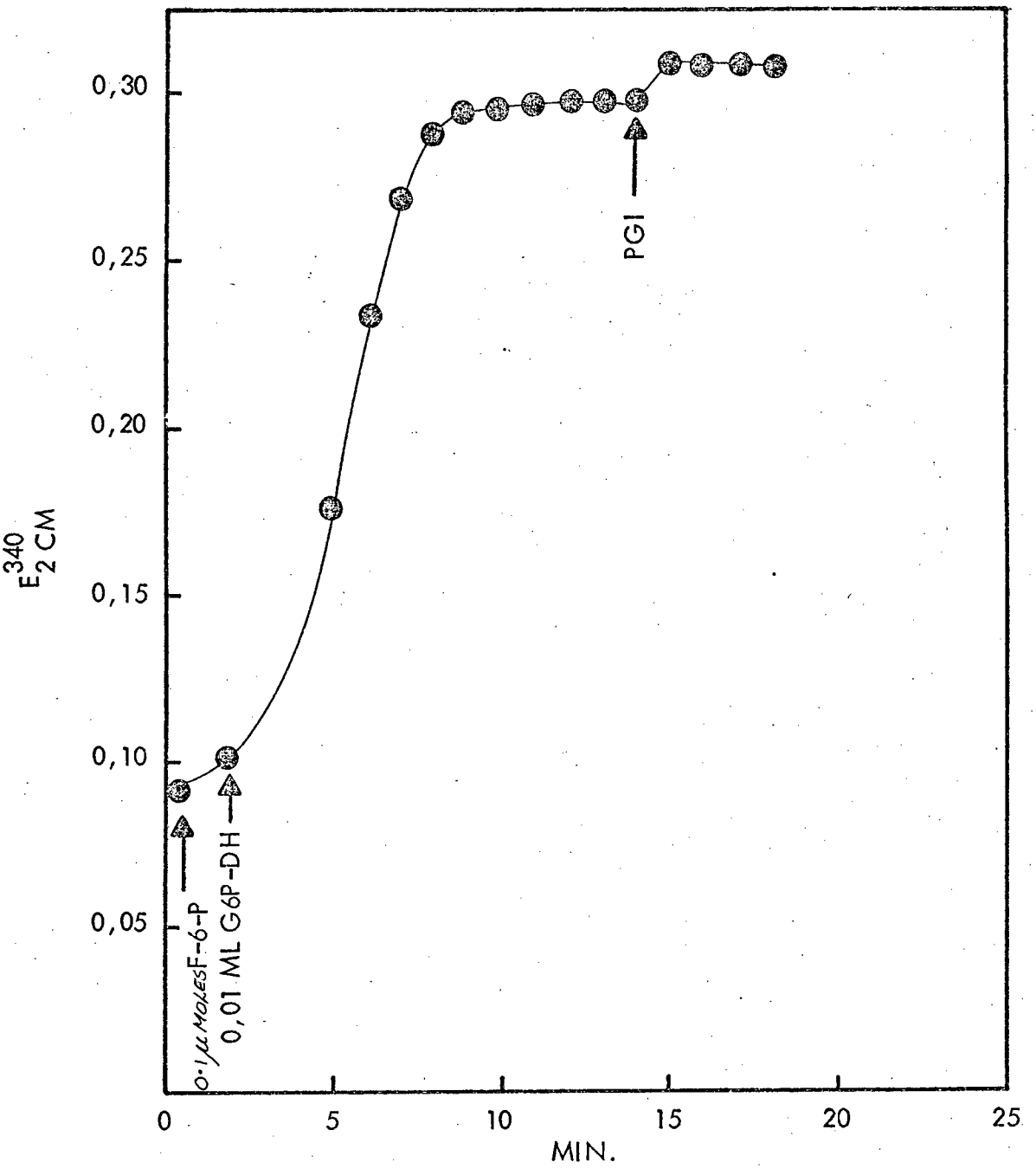


FIG. 6. DETERMINATION OF PURITY OF G6P-DH FOR F-6-P ASSAY. DURING ASSAY PROCEDURE F-6-P, G6P-DH AND PGI ARE ADDED AS SHOWN. MAJOR INCREASE IN O.D. OCCURRED BEFORE PGI WAS ADDED INDICATING CONTAMINATION OF G6P-DH BY SIGNIFICANT QUANTITIES OF PGI.

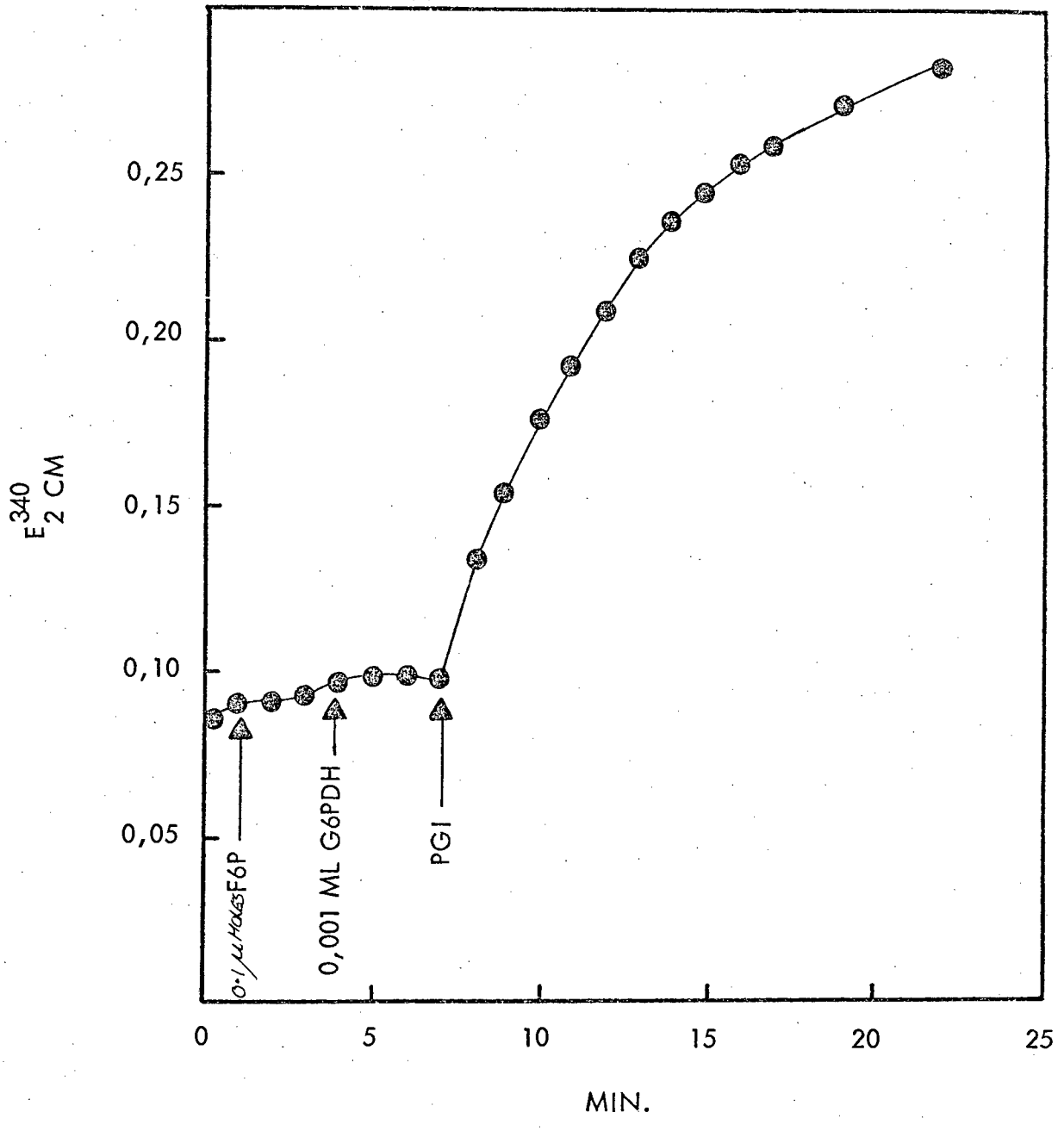


FIG. 7. EXPERIMENTAL PROCEDURE AS IN FIG. 6, BUT LESS G6P-DH USED. NO SIGNIFICANT INCREASE IN O.D. OCCURRED BEFORE PGI ADDED.

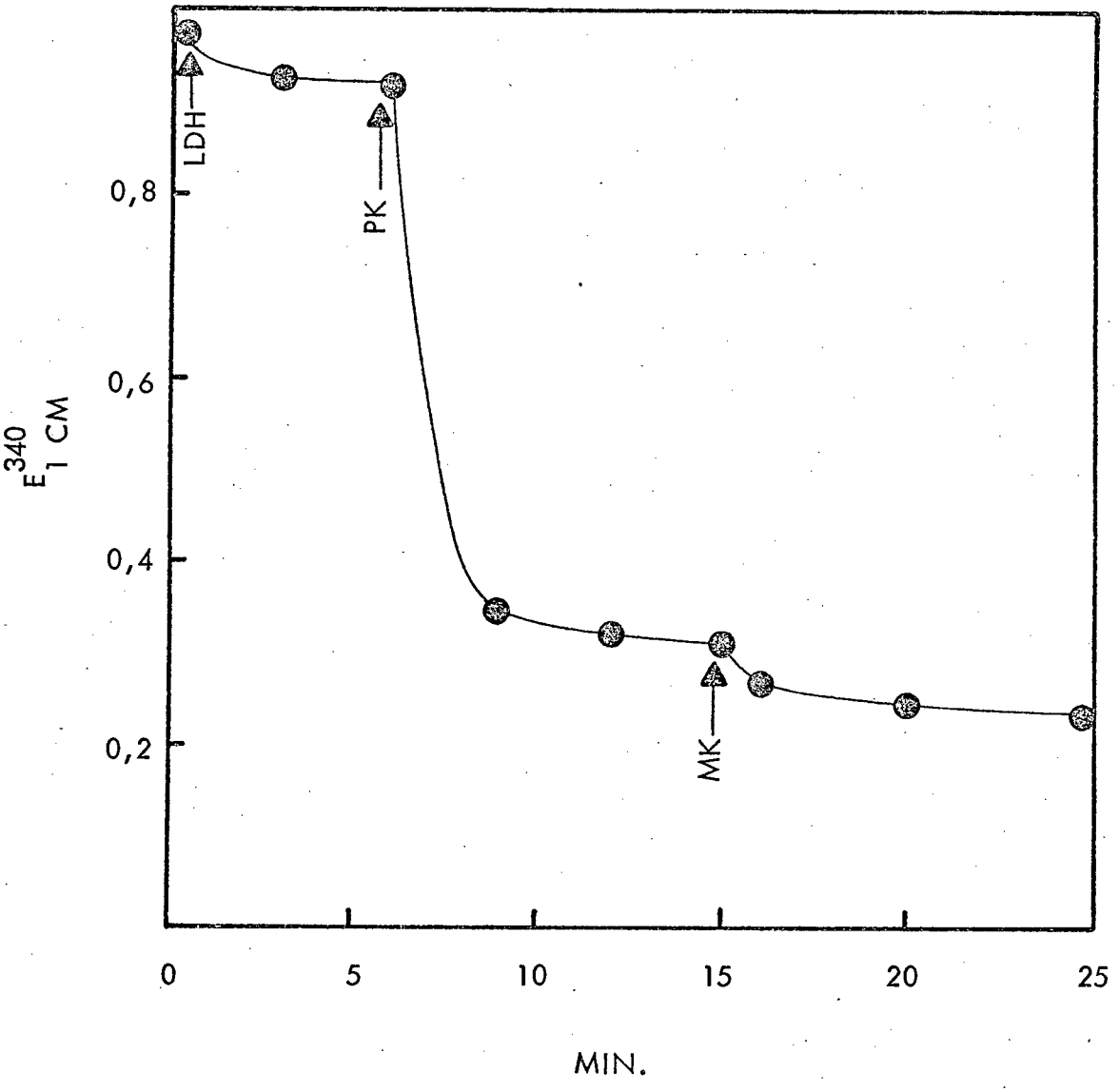


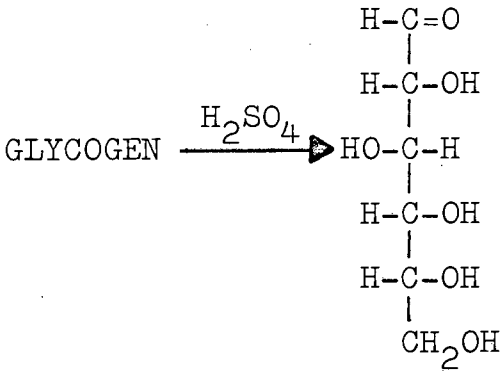
FIG. 8. DETERMINATION OF PYRUVATE, ADP AND AMP IN THE PCA EXTRACT OF MUSCLE (PIG 22 B; +12½ MIN.)

V. DETERMINATION OF GLYCOGEN

(TÁRNOKY and NAGY, 1963)

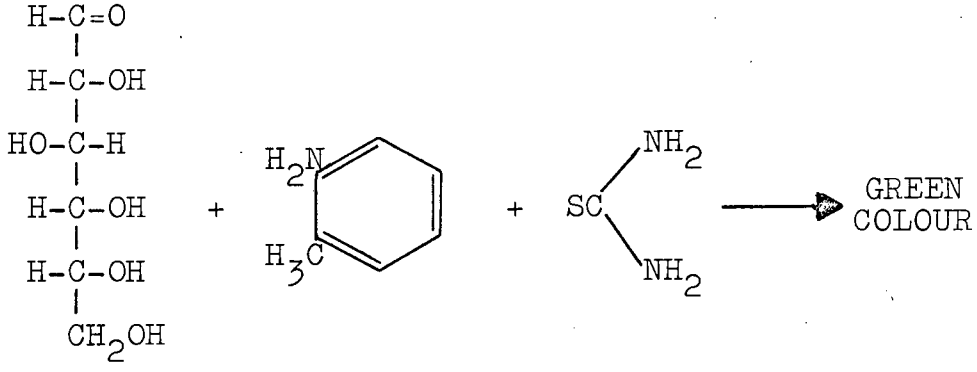
Principle:

(1)



GLUCOSE

(2)



GLUCOSE O-TOLUIDINE THIOUREA

Glycogen was extracted with trichloro-acetic acid, precipitated with ethanol and then hydrolysed with H_2SO_4 to glucose. Glucose was then determined by the o-toluidine-

glucose coupling reaction, which is specific for the aldohexoses. Thiourea stabilizes the reagent, lowers the blank reading to almost zero and produces a pure green-blue colour in the presence of glucose (HYVÄRINEN and NIKKILÄ, 1962).

Preparation of the TCA extract

Muscle samples were frozen within 5 to 8 seconds after removal by immersing immediately into liquid nitrogen. The frozen tissue was homogenized in cold TCA (10% w/v) with an Ultra Turrax homogenizer in the cold (4°C). The homogenate was brought to 30 ml per gram of tissue with 10% TCA, and centrifuged at 1 000 x g for 10 minutes in a refrigerated Sorvall RC-2B centrifuge.

Assay Procedure

Absolute ethanol (2 vol.) was added to 2,0 ml of the supernatant, and the mixture

left overnight in the cold (4°C). After centrifuging at 1 000 x g for 20 minutes in a refrigerated Sorvall RC-2B centrifuge, the supernatant was carefully decanted and the precipitate dried by placing the tubes in an inverted position on tissue paper. The precipitate was dissolved in 0,5 ml of 2 N H_2SO_4. The tubes, each stoppered with a marble, were transferred to a boiling water bath, and boiled for one hour. After cooling, 8,0 ml of o-toluidine reagent (toluidine (6% v/v) and thiourea (0,15% w/v) in glacial acetic acid) was added. The whole was well mixed and boiled again for 8 minutes. A green colour developed, and after cooling, the O.D. was measured against air at 635 nm. A blank and glucose standard containing 500 µg glucose in 2,0 N H_2SO_4 were also prepared.

Calculation/ .....

Calculation

$$\frac{\Delta E}{\Delta E_{STD}} \times \frac{V_{STD}}{M} \times \frac{1}{2} = \mu\text{moles/ml (glucose equivalents)}$$

μMOLES/ML x DILUTION = μMOLES/G.

ΔE_{STD} = O.D. reading of the standard

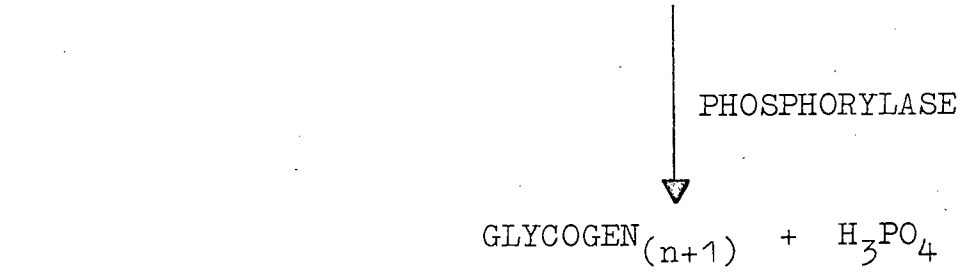
V_{STD} = value of the standard (= 500 μg)

M = Molecular weight of glucose (= 180)

VI. DETERMINATION OF MUSCLE PHOSPHORYLASE

(HERS, 1964)

Principle:



Phosphorylase was measured by the liberation of inorganic phosphate from glucose-1-phosphate in the presence of glycogen. This reaction is the reverse of the process operating *in vivo* (HERS, 1964). Phosphorylase exists in two forms, namely phosphorylase *a*, also known as the active form and phosphorylase *b*, the inactive form. Phosphorylase *a* has 60-70% of its maximum activity in the absence of AMP, and phosphorylase *b* has an absolute requirement for AMP (CORI, CORI and GREEN, 1943). To determine the total phosphorylase activity, i.e. phosphorylase *a* and *b*, the assay was carried out in the presence of AMP. To measure the amount of phosphorylase *b* present, the assay was performed in the absence of AMP.

Reagents

1. The substrate mixture consists of glucose-1-phosphate, 0,1M, glycogen, 2%, sodium fluoride, 0,2M with ~~or~~ and without AMP, 0,003M. The pH was adjusted to 6,1 with 1N HCl.

2. The Fiske-Subbarow reagent consists of 1-amino-2-naphthol-4-sulfonic acid, 0,5 g, sodium bisulfite, 30 g (anhydrous) and sodium sulfite, 1 g (anhydrous). The sodium bisulfite was dissolved in 200 ml of water. The other chemicals were added and stirred well to dissolve them. The reagent was filtered and stored in the dark. It will keep for about 10 days.

Preparation of Tissue Extract

The muscle samples were frozen within 5 to 8 seconds after removal by immersing it immediately into liquid nitrogen. The frozen tissue was homogenized in the cold ($4^{o}C$) in cold doubly-distilled water with an Ultra

Turrax homogenizer. The homogenate was diluted to a concentration of 1% (w/v) in cold doubly-distilled water and centrifuged at 1 000 x g for 10 minutes in a refrigerated Sorvall RC-2B centrifuge.

Assay Procedure

Substrate containing AMP (0,05 ml) and substrate containing no AMP (0,05 ml) were pipetted into separate test tubes. 0,05 ml of a 1% tissue extract was added. A blank containing 0,05 ml of water, and a standard containing 0,5 µmoles of phosphate, instead of the tissue extract, was included. The preparations were incubated at 37°C in a water bath for 0, 5, 10 and 20 minutes. The reaction was terminated by the addition of 0,5 ml of 1N TCA. Then, in turn, were added 0,25 ml 5% ammonium molybdate, 0,25 ml 10N H_2SO_4 and 3,7 ml distilled water. The final volume was then 4,8 ml. After mixing well, 0,2 ml Fiske-Subbarow reagent was added and the solution left at room temperature for 10 minutes to

allow development of molybdate blue. O.D. was measured at 660 nm in a Zeiss PMQII spectrophotometer.

Calculation

Factor x ΔE = µmoles of phosphate liberated per ml.

Factor x ΔE x 100 = µmoles of phosphate liberated per g.

ΔE = reading of test - blank.

$$\text{Factor} = \frac{S}{E_S} \times 20$$

where S = value of the standard

E_S = reading of the standard

20 = dilution factor

Phosphorylase activity was expressed in terms of inorganic phosphate liberated/ g. tissue/ minute.

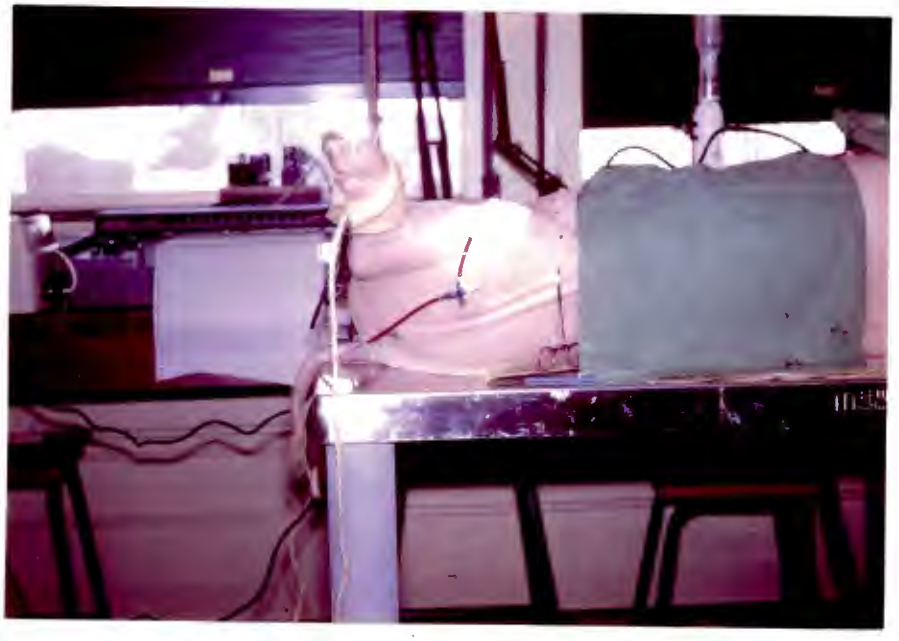
PART III

R E S U L T S

Pigs 22B and 139 developed sustained rigor. Pig 137 was anomalous in that it did not develop rigor, but pyrexia was typical. Although Pig 137 presented no evidence of rigor after succinyl choline administration, glycolysis was nevertheless accelerated. Succinyl choline in this animal produced the usual type of response, i.e. a generalized muscular contraction, due to membrane depolarization, which lasted for 2 to 3 seconds in this animal. Immediately afterwards, skeletal muscle in this pig relaxed in contrast to animals 22B and 139 in which rigor was persistent. (Figs. 9 and 10).

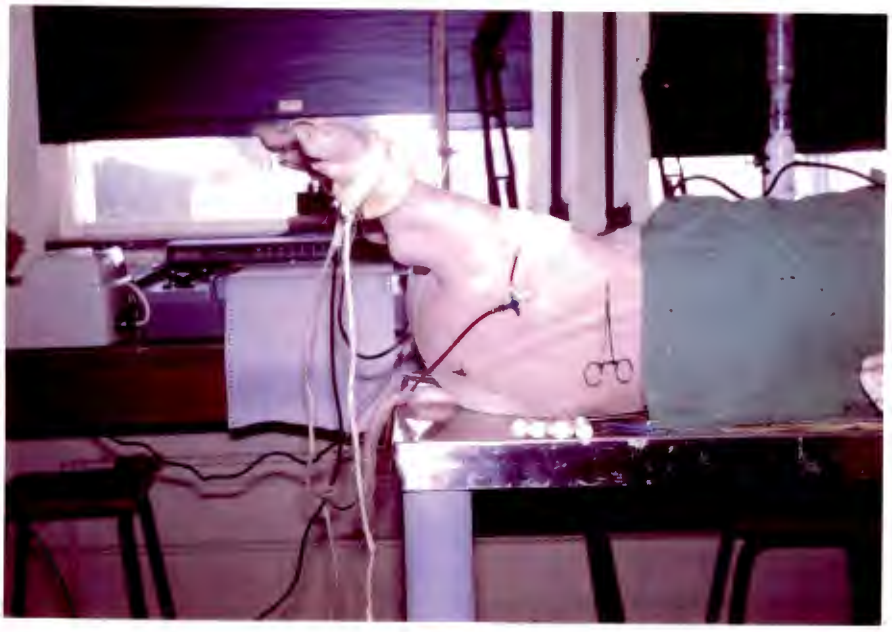
All three pigs developed systemic lactacidosis. In Pig 137, however, its degree was less severe than in the other two animals. Blood lactate concentration of Pig 137 was about 5 to 6 times less than the other animals. Serial alterations in blood lactate concentrations are shown in Figs. 11 a, b and c. Accumulation of muscle lactate in Pig 83C is correlated with a fall in muscle glycogen (Fig. 12). The fact that the curves show the expected 2:1 stoichiometry between lactate and glycogen glucose equivalents is fortuitous since lactate is being continuously removed by blood circulation.

FIG. 9.



HIND LIMBS OF HALOTHANE SUSCEPTIBLE PIG DURING CONTROL THIOPENTONE ANAESTHESIA. THE LIMBS ARE FLACCID.

FIG. 10.



SAME PIG AS IN FIG. 9. 10 MIN. AFTER HALOTHANE.
ALL FOUR LIMBS BECAME EXTREMELY RIGID.

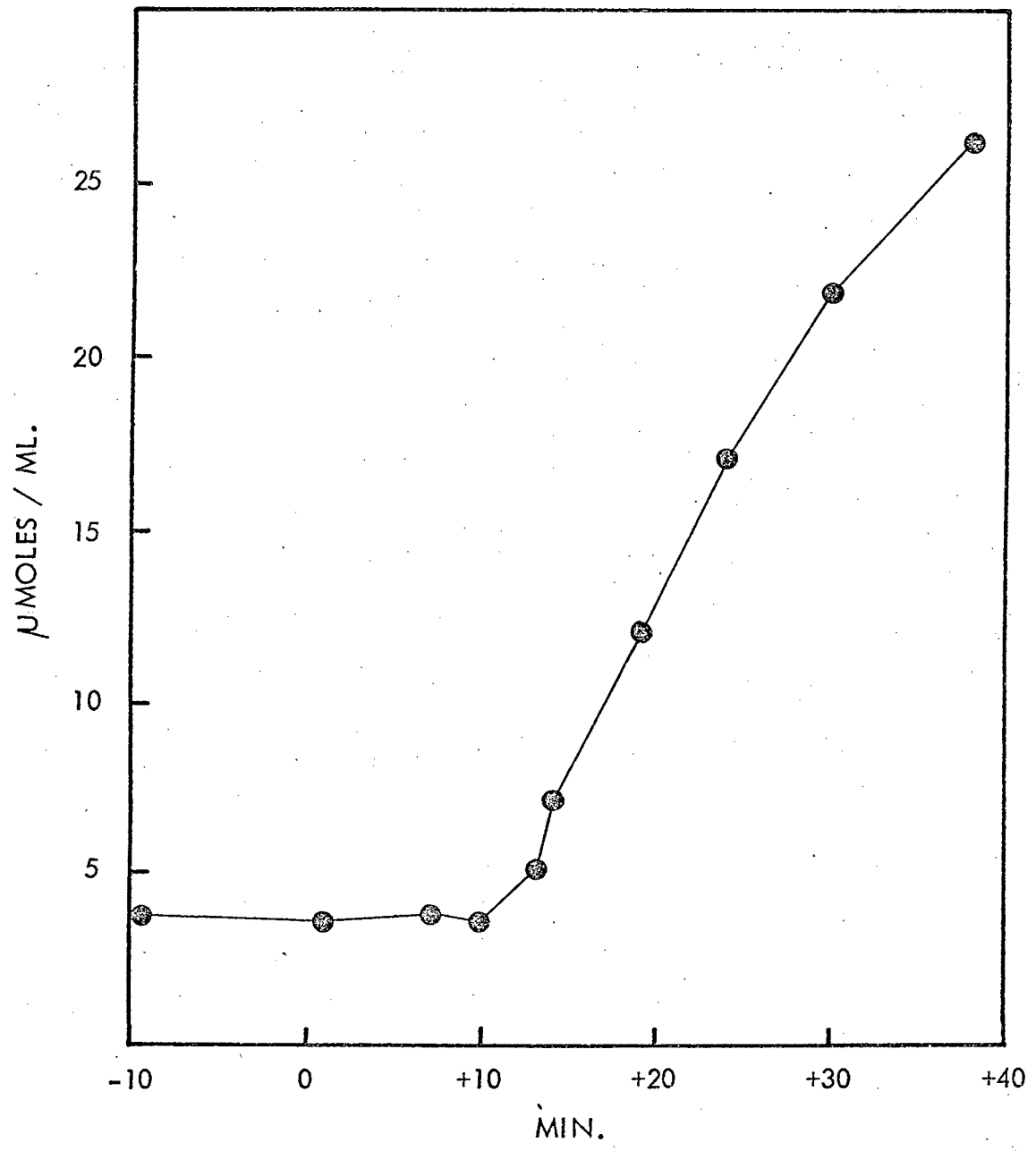


FIG. 11a. PIG 22B. BLOOD LACTATE. HALOTHANE ADMINISTERED AT 0 MIN. AND SUCCINYL CHOLINE AT 12 MIN.

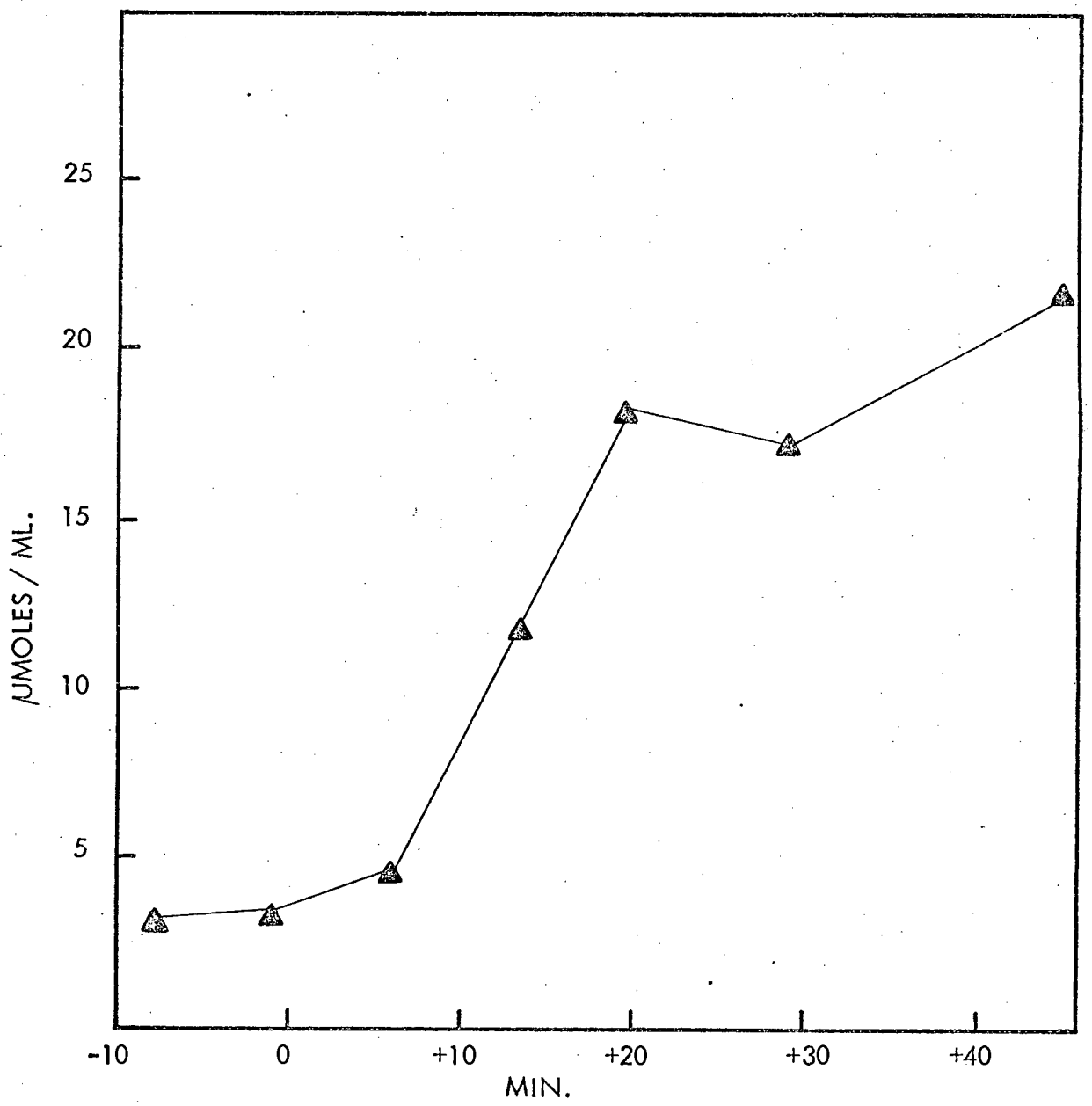


FIG. 11b. PIG 139. BLOOD LACTATE. HALOTHANE ADMINISTERED AT 0 MIN. AND SUCCINYL CHOLINE AT 10 MIN.

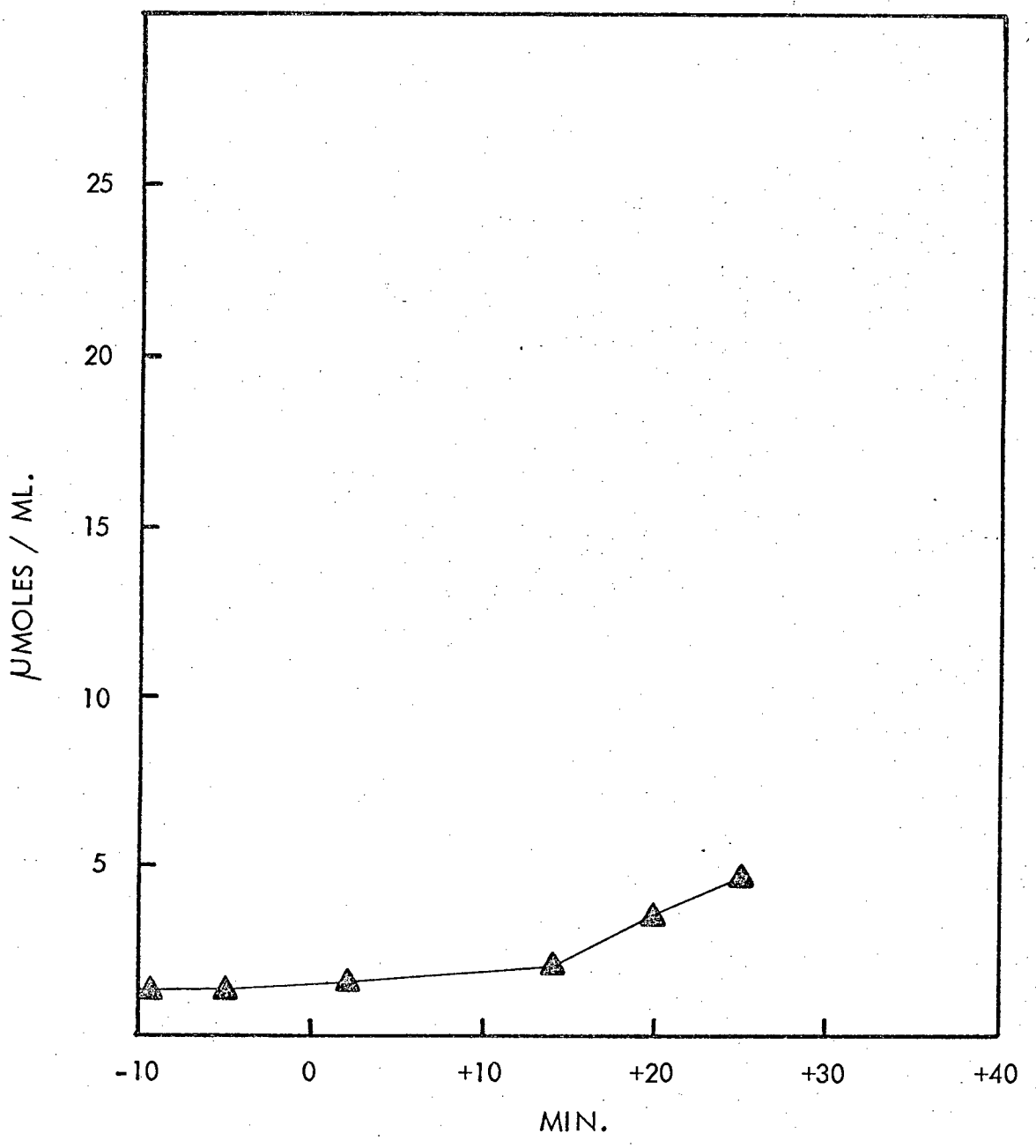


FIG. 11c. PIG 137. BLOOD LACTATE. HALOTHANE ADMINISTERED AT 0 MIN. AND SUCCINYL CHOLINE AT 14 MIN.

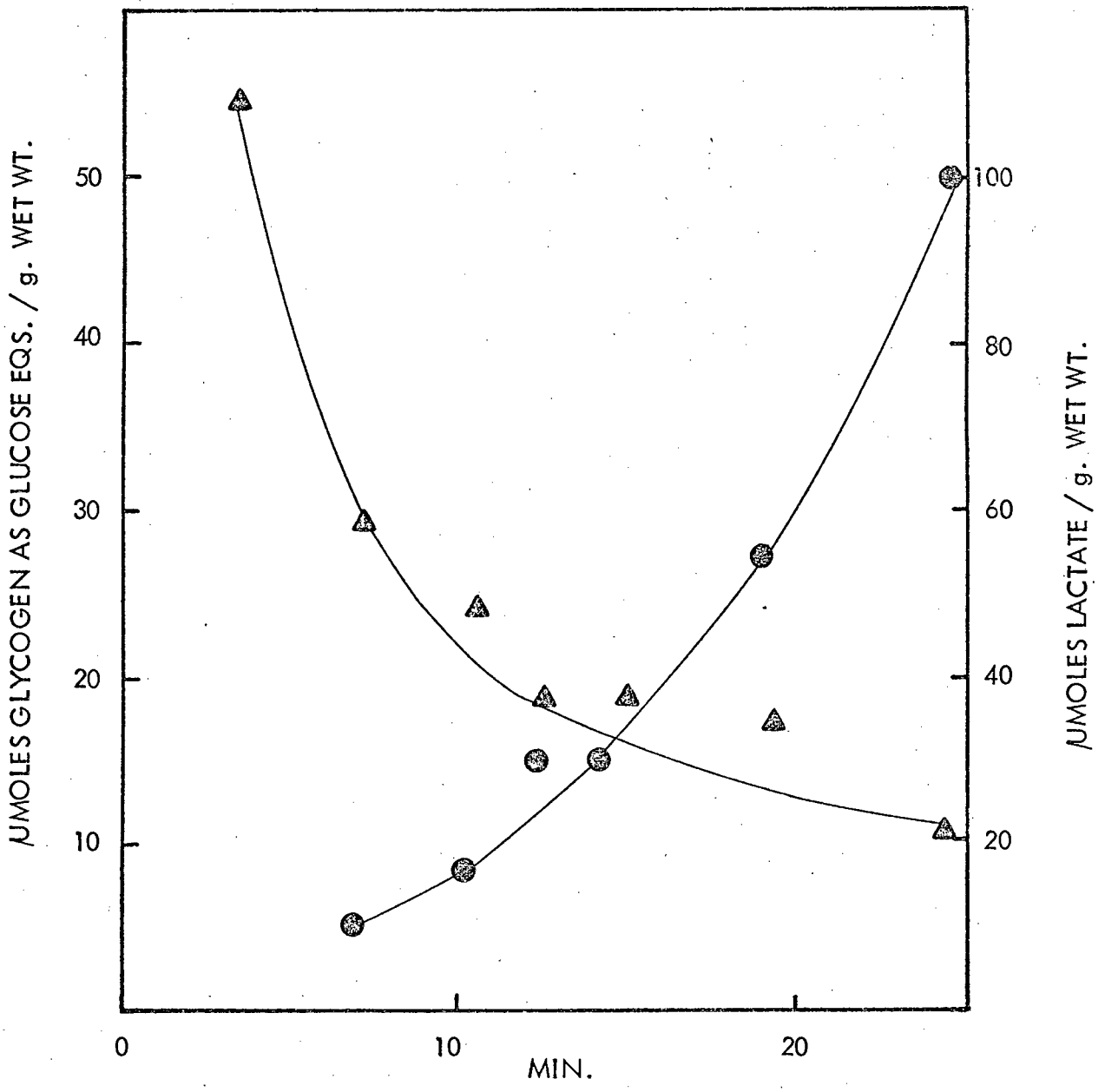


FIG. 12. PIG 83 C. MUSCLE GLYCOGEN AND LACTATE CONTENT DURING INDUCTION OF HYPERTHERMIA WITH HALOTHANE (3% V/V) ADMINISTERED FROM 0 MIN.

▲—▲ GLYCOGEN ●—● LACTATE

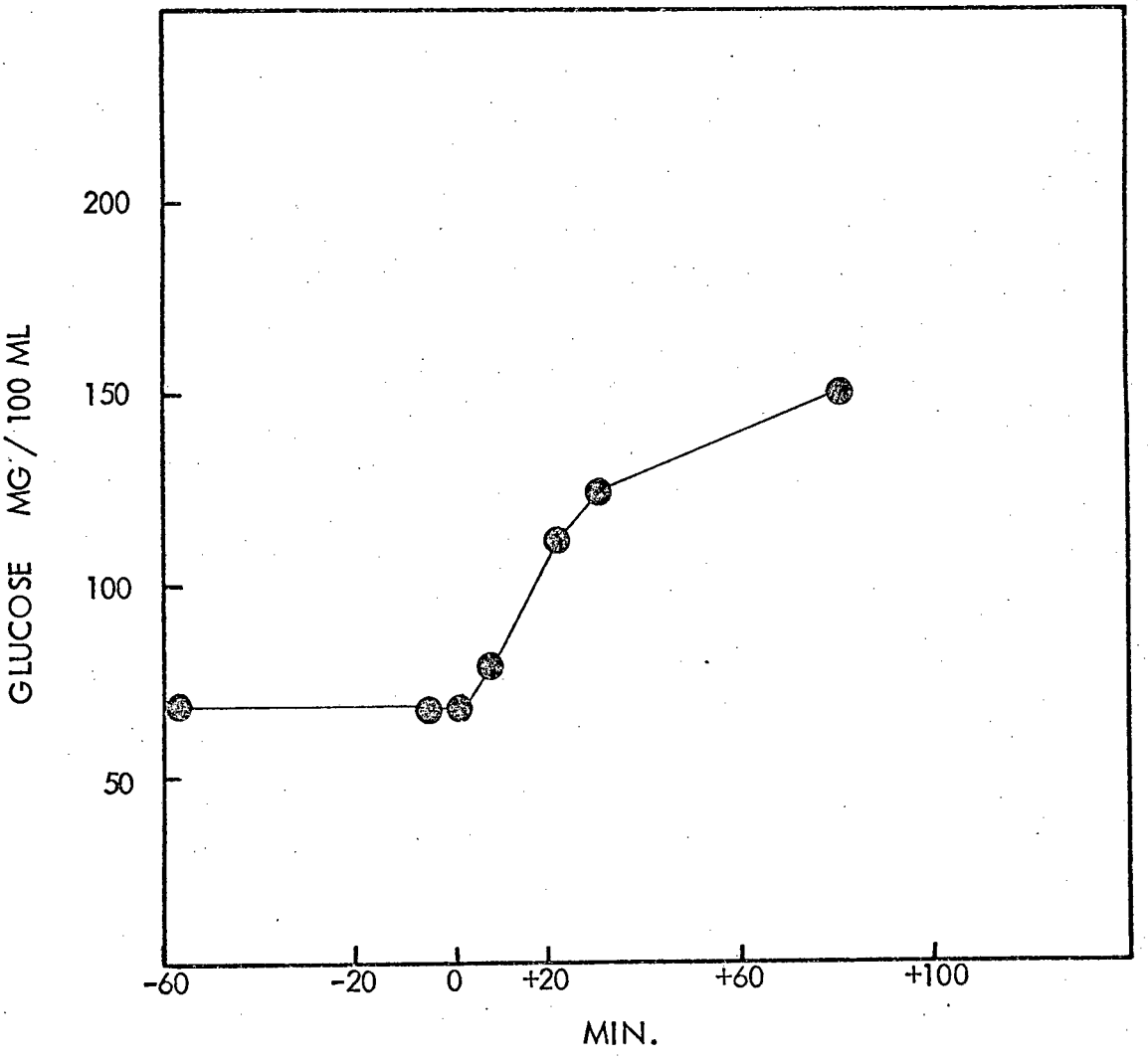


FIG. 13. PIG 83C. BLOOD GLUCOSE DURING INDUCTION OF MALIGNANT HYPERTHERMIA BY HALOTHANE. HALOTHANE (3% V/V) ADMINISTERED FROM 0-60 MIN.

As seen in Fig. 12 there was a fall in glycogen when the concentrations of lactate in muscle rose. Blood glucose became elevated (Fig. 13).

Determination of the glycolytic intermediates was done in duplicate on each sample, and the average of the two readings was taken. Discrepant results were repeated, also in duplicate. Tables II, III and IV represent the data on the glycolytic intermediates and cofactors determined in Pigs 22B, 139 and 137. In all the animals, following administration of succinyl choline, there was a rise in muscle lactate which moved in parallel with the rise in blood lactate, with little or no alteration after halothane. The behaviour of individual compounds is also shown in Figs. 14 a to h for Pig 22B; 15 a to h for Pig 139 and 16 a to h for Pig 137. In order to localize possible points of glycolytic control in the system, the ratios of glycolytic intermediate pairs have been calculated (Tables V, VI and VII and Figs. 17 a to f) and compared with equilibrium constants determined by WILLIAMSON, 1965.

To establish whether the method used for estimating activation of phosphorylase b to a was satisfactory an experiment was performed on

rat skeletal muscle. Portions of muscle were taken before and after the administration of a lethal intravenous dose of adrenaline and the phosphorylase assays were performed on each sample. Adrenaline should increase the percentage of phosphorylase in the a form, since it stimulates the conversion of phosphorylase b to a. In this experiment, phosphorylase a increased from 12 to 48% (Fig. 18).

Fig. 19 shows the alteration in total phosphorylase activity during a typical malignant hyperthermic episode in Pig R101 which in this instance was induced by halothane alone. There is fluctuation in the total activity with a peak immediately after introducing halothane. A similar pattern has been noted in one other pig as well as in a normal control. The exact significance of total phosphorylase activity is, however, not established since levels noted by others show wide fluctuations and most authors express phosphorylase a as a percentage of phosphorylase b instead of as absolute activity.

TABLE II

GLYCOLYTIC INTERMEDIATES AND COFACTORS IN SKELETAL MUSCLE (PIG 22B)

(/UMOLES/G. WET WT.)

MIN	-10	+1	+7	+10	+13	+14	+19	+24	+30	+38
GLUCOSE-1-PHOSPHATE	0,084	0,084	0,084	0,074	0,168	0,213	0,232	0,274	0,271	0,252
GLUCOSE-6-PHOSPHATE + FRUCTOSE-6-PHOSPHATE	0,784	0,983	0,835	0,643	1,50	1,99	1,75	2,14	3,11	2,93
FRUCTOSE-1,6-DIPHOSPHATE	0,713	0,633	0,534	0,641	0,891	0,846	1,06	1,54	1,30	1,90
DIHYDROXYACETONE PHOSPHATE	0,058	0,057	0,045	0,051	0,080	0,075	0,099	0,120	0,123	0,135
∝ -GLYCEROL PHOSPHATE	0,463	0,510	0,565	0,458	0,697	0,830	0,622	0,690	1,028	1,048
GLYCERALDEHYDE-3-PHOSPHATE	0,003	0,004	0,004	0,003	0,005	0,004	0,008	0,014	0,006	0,009
3-PHOSPHOGLYCERATE	0,174	0,189	0,202	0,180	0,270	0,262	0,182	0,240	0,272	0,252
2-PHOSPHOGLYCERATE	0,052	0,053	0,066	0,063	0,073	0,076	0,098	0,103	0,079	0,097
PHOSPHOENOLPYRUVATE	0,073	0,074	0,073	0,068	0,087	0,084	0,066	0,090	0,082	0,079
PYRUVATE	0,125	0,120	0,115	0,107	0,157	0,197	0,185	0,140	0,134	0,120
LACTATE	9,17	8,63	7,21	7,45	10,64	13,61	14,87	13,74	15,58	13,48
MALATE	0,058	0,068	0,066	0,073	0,045	0,085	0,066	0,113	0,129	0,131
ADENOSINE-5'-MONOPHOSPHATE	0,115	0,157	0,208	0,160	0,207	0,197	0,098	0,191	0,212	0,256
ADENOSINE-5'-DIPHOSPHATE	0,565	0,701	0,816	0,711	0,947	0,704	0,864	1,09	1,08	0,882
ADENOSINE-5'-TRIPHOSPHATE	5,15	4,86	5,61	5,25	5,26	5,05	5,22	5,79	5,73	5,72
CREATINE PHOSPHATE	11,35	9,67	14,11	11,89	11,74	12,01	11,69	11,63	11,22	9,59

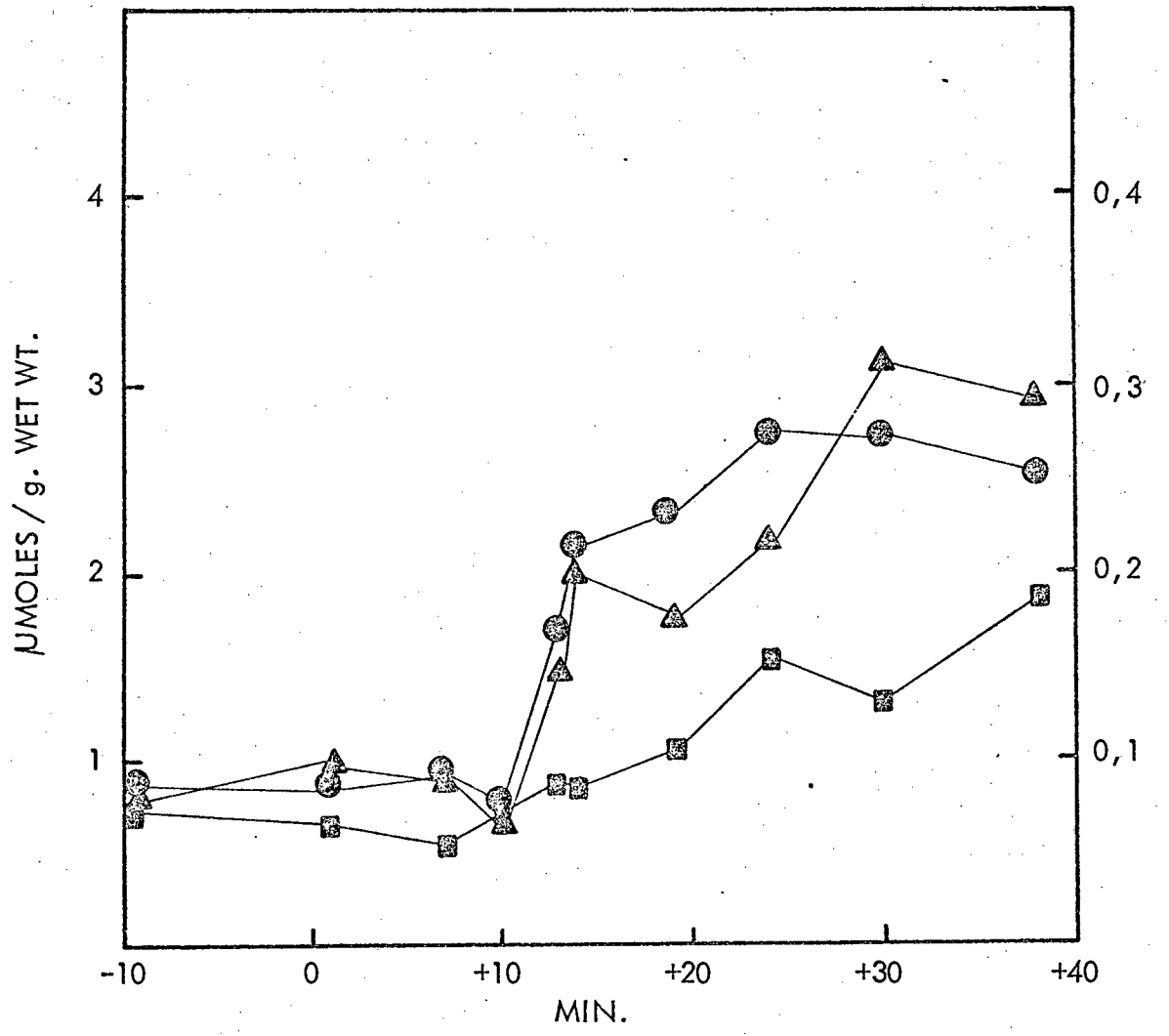


FIG. 14a. PIG 22B. MUSCLE G-1-P (●——●)
G-6-P + F-6-P (▲——▲) AND FDP (■——■).
HALOTHANE ADMINISTERED AT 0 MIN. AND
SUCCINYL CHOLINE AT 12 MIN.

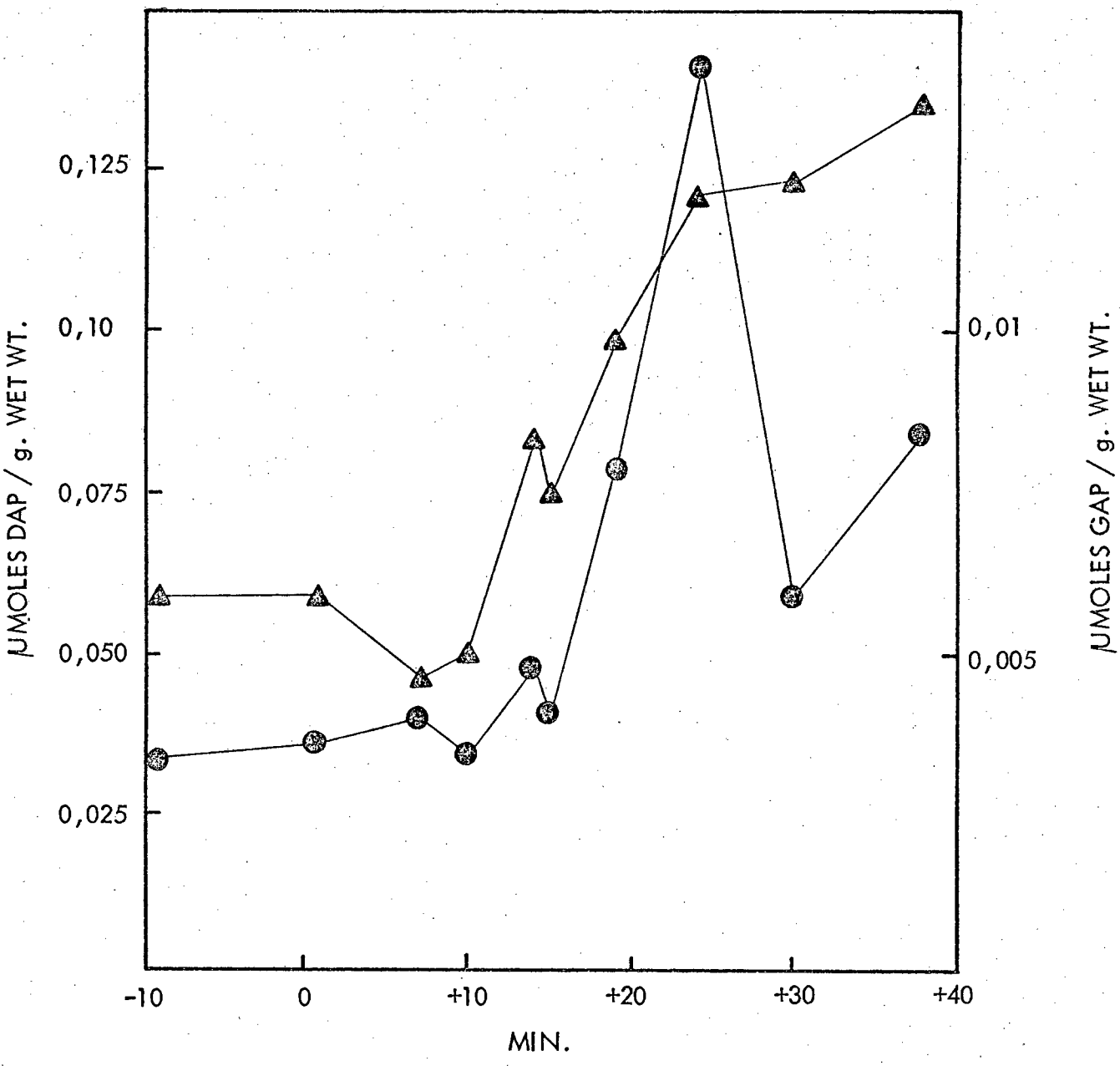


FIG. 14b. PIG 22B. MUSCLE DAP (▲——▲) AND GAP (●——●). EXPERIMENTAL DETAILS AS IN FIG. 14a.

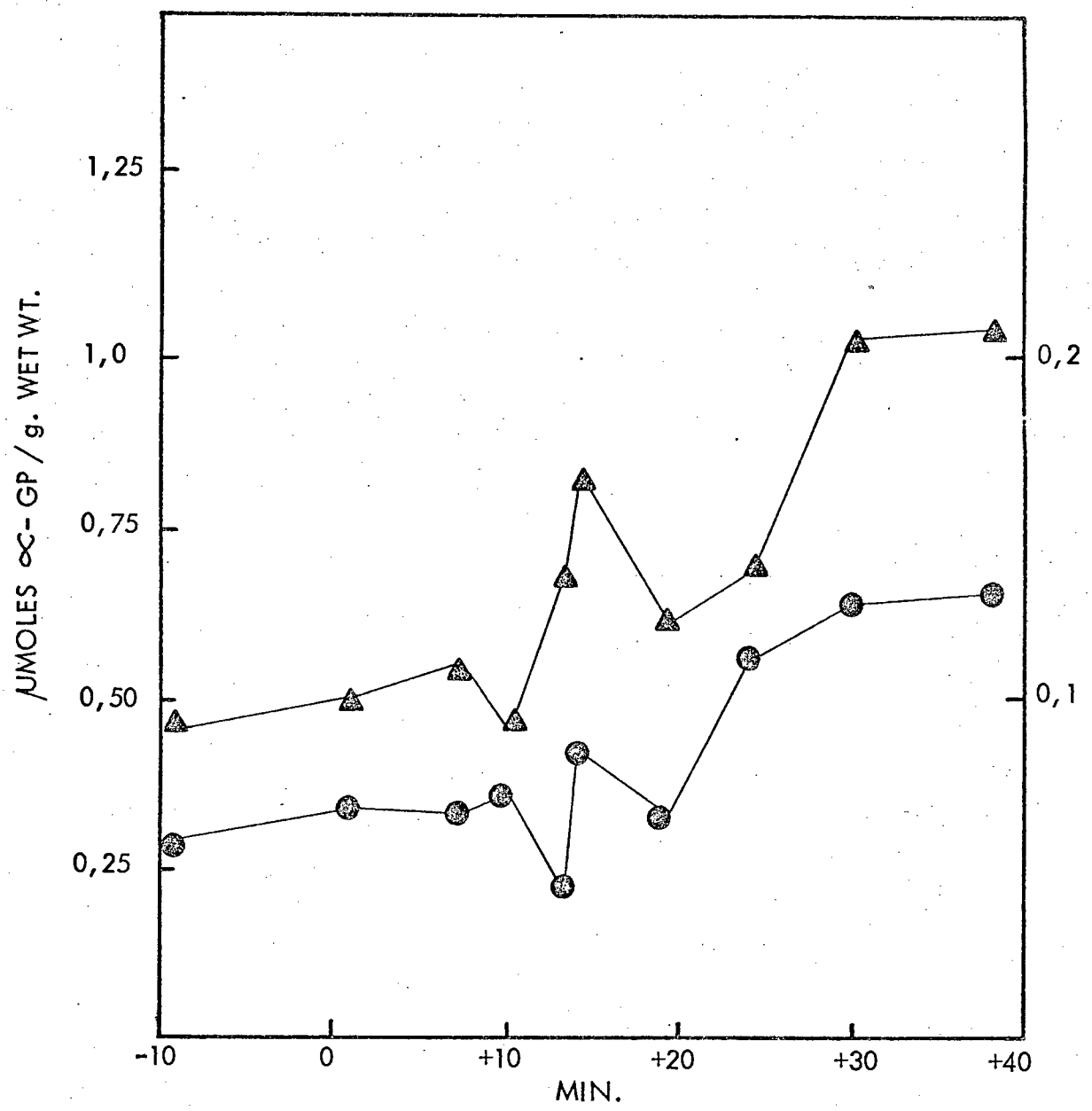


FIG. 14c. PIG 22B. MUSCLE ∝-GP (▲——▲) AND MALATE (●——●). EXPERIMENTAL DETAILS AS IN FIG. 14a.

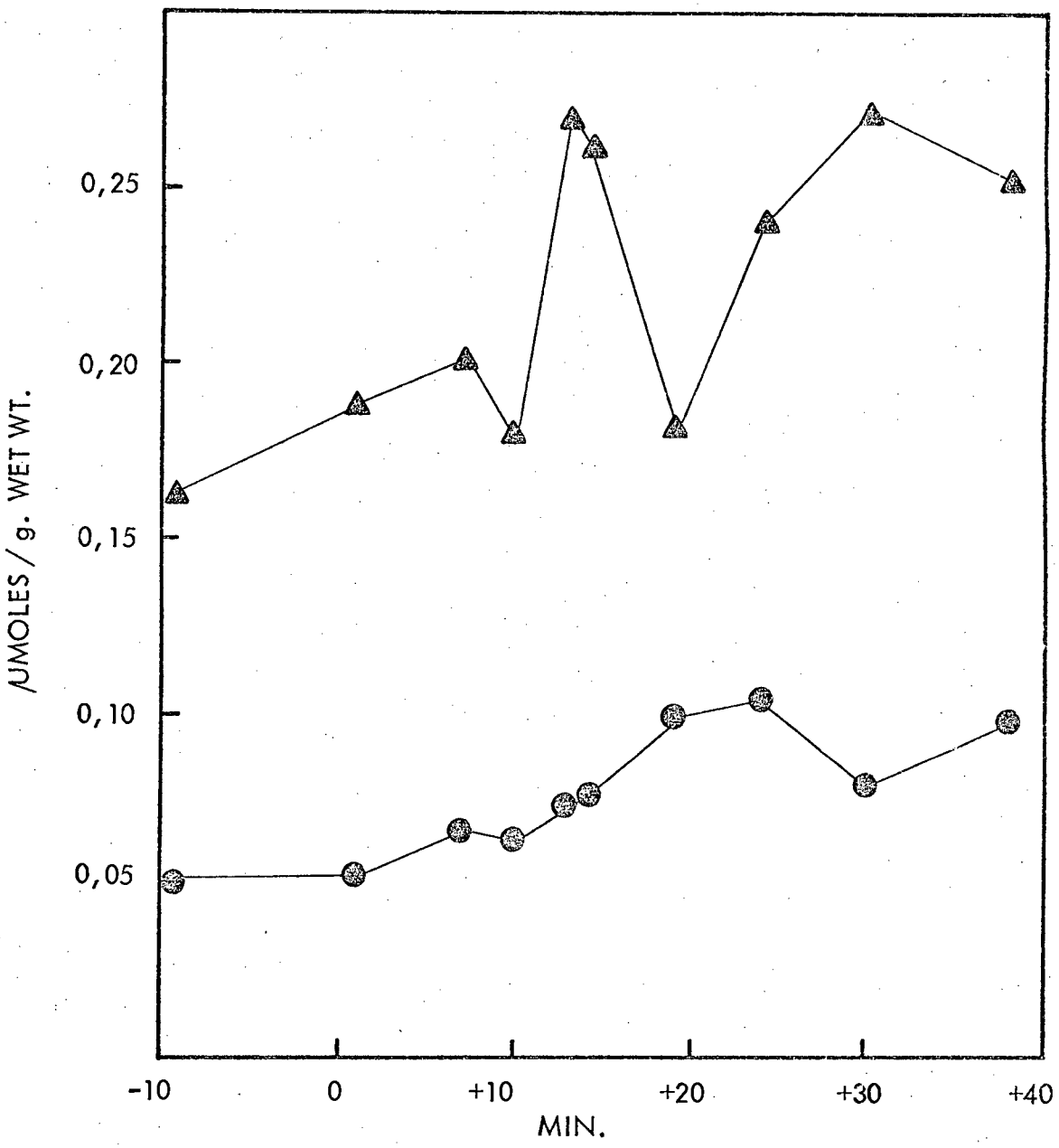


FIG. 14d. PIG 22B.
MUSCLE 3-PG (▲——▲) AND 2-PG (●——●).
EXPERIMENTAL DETAILS AS IN FIG. 14a.

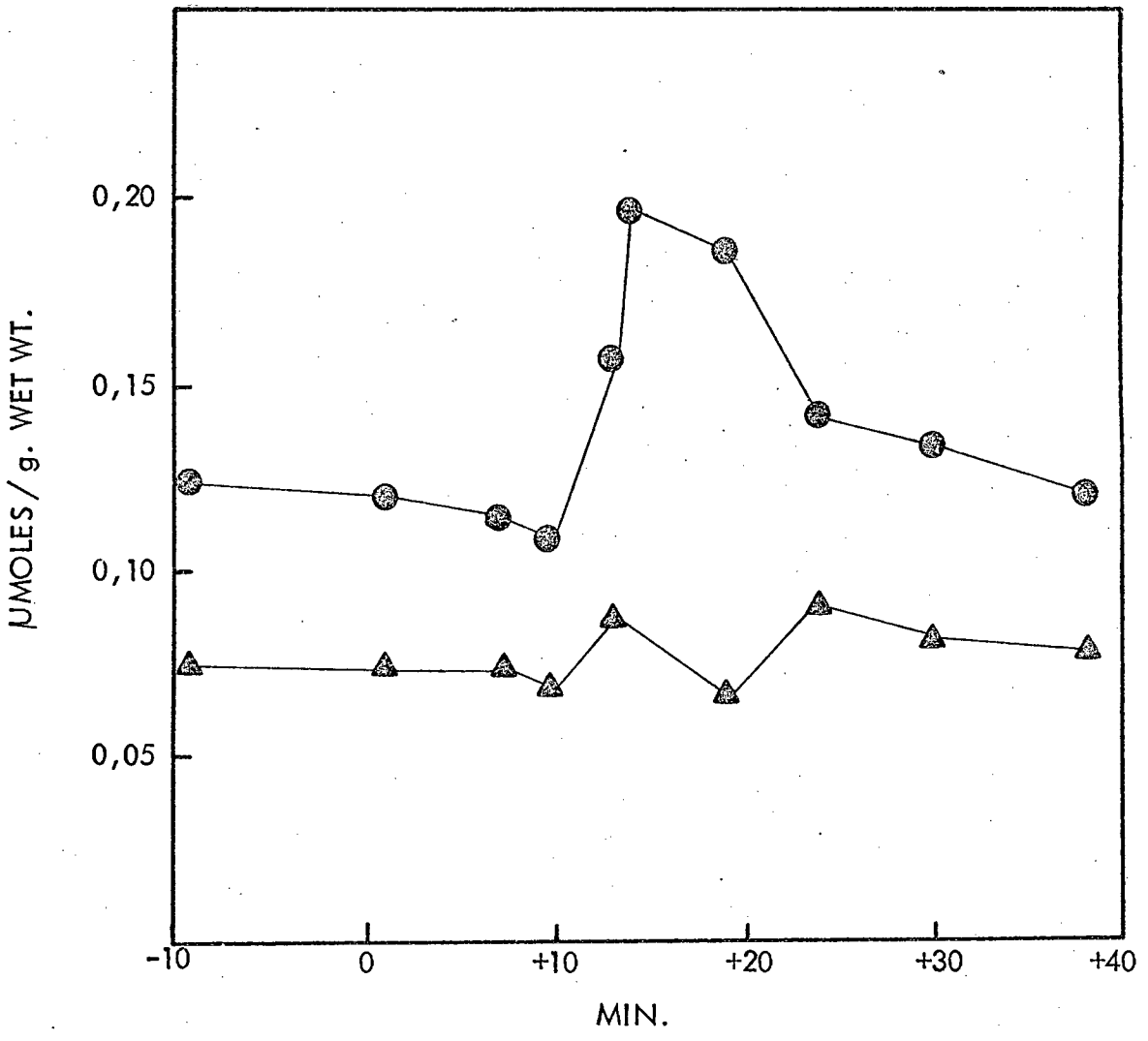


FIG. 14e. PIG 22 B. MUSCLE PEP (▲——▲) AND PYRUVATE (●——●). EXPERIMENTAL DETAILS AS IN FIG. 14a.

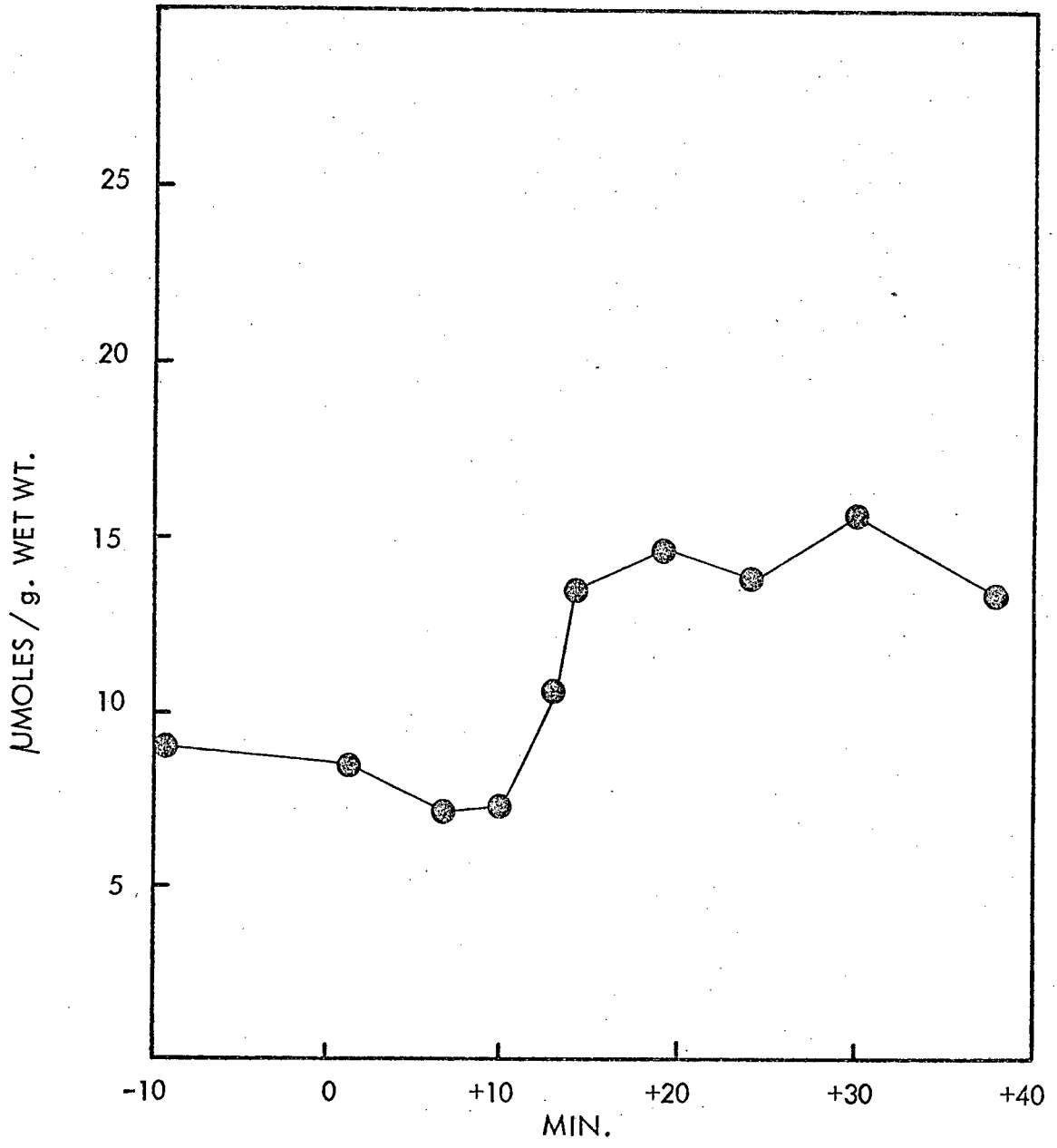


FIG. 14f. PIG 22B. MUSCLE LACTATE. EXPERIMENTAL DETAILS AS IN FIG. 14a.

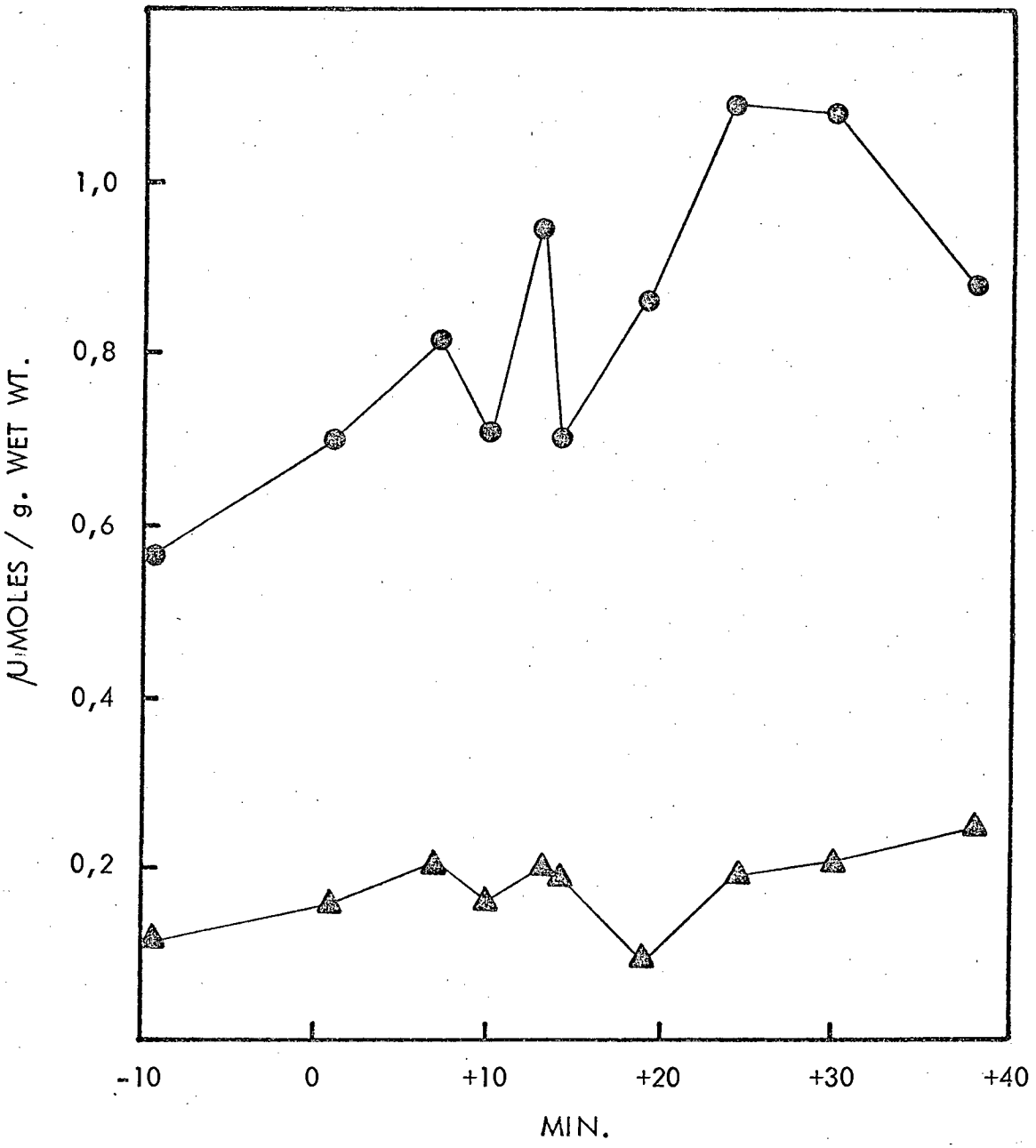


FIG. 14 g. PIG 22B. MUSCLE AMP (▲——▲) AND ADP (●——●). EXPERIMENTAL DETAILS AS IN FIG. 14a.

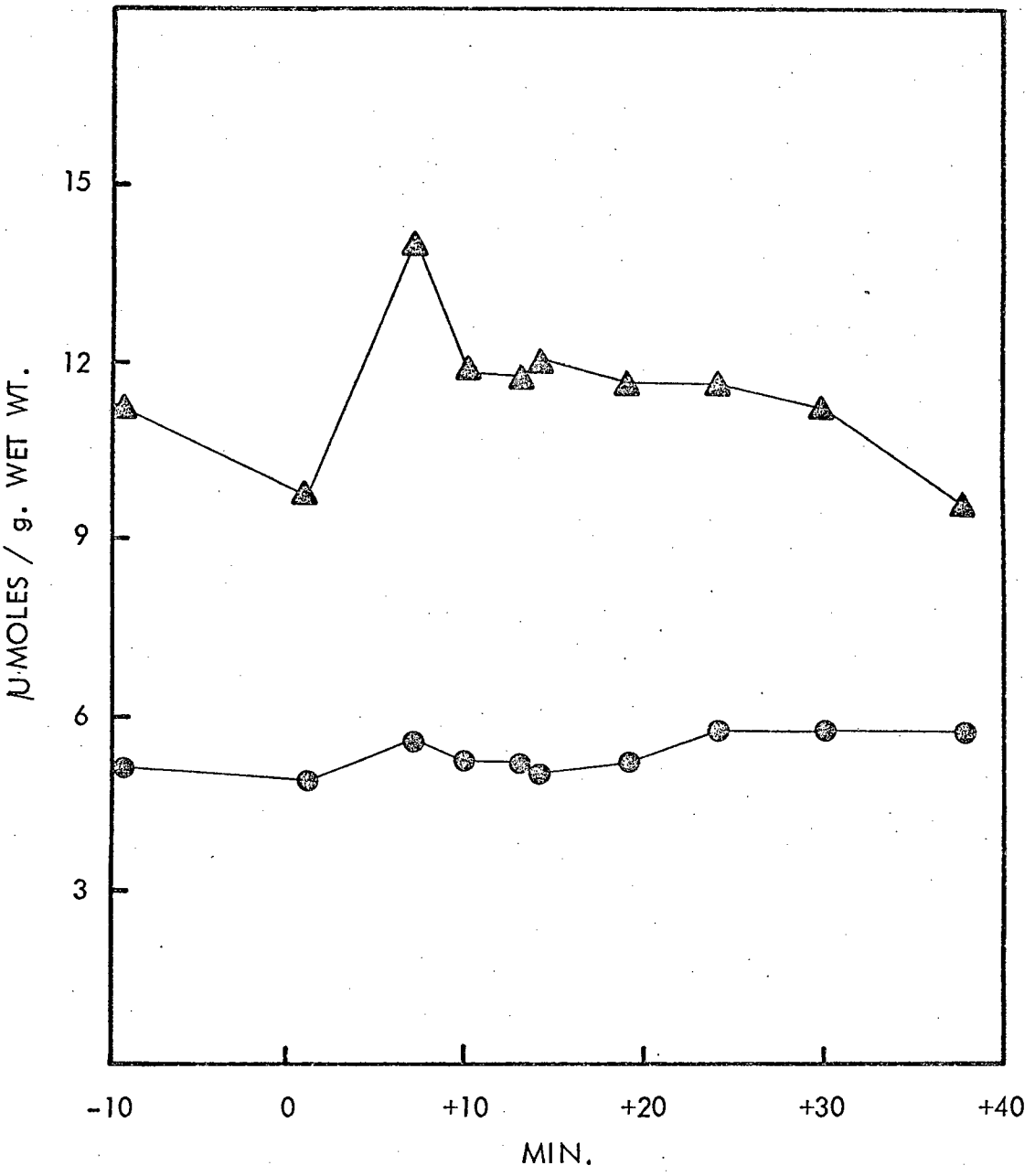


FIG. 14h. PIG 22B. MUSCLE ATP (●——●) AND CP (▲——▲). EXPERIMENTAL DETAILS AS IN FIG. 14a.

TABLE III

GLYCOLYTIC INTERMEDIATES AND COFACTORS IN SKELETAL MUSCLE (PIG 139)

(UMOLES/G. WET WT.)

MIN	-10	-5	+4	+12½	+17½	+25	+35	+45
GLUCOSE-1-PHOSPHATE	0,074	0,098	0,220	0,199	0,486	0,421	0,179	0,268
GLUCOSE-6-PHOSPHATE	0,584	0,752	1,56	1,71	3,55	3,75	2,00	2,81
FRUCTOSE-6-PHOSPHATE	0,081	0,098	0,087	0,151	0,426	0,414	0,194	0,367
FRUCTOSE-1,6-DIPHOSPHATE	0,221	0,357	0,546	0,525	1,43	1,25	1,14	1,43
∝-GLYCEROL PHOSPHATE	0,235	0,304	0,571	0,690	1,08	1,12	0,624	1,11
3-PHOSPHOGLYCERATE	0,129	0,151	0,289	0,264	0,212	0,214	0,172	0,259
2-PHOSPHOGLYCERATE	0,015	0,016	0,042	0,037	0,042	0,034	0,021	0,045
PHOSPHOENOLPYRUVATE	0,092	0,071	0,040	0,042	0,037	0,031	0,043	0,045
PYRUVATE	0,095	0,089	0,172	0,137	0,267	0,220	0,089	0,098
LACTATE	6,30	7,09	9,90	10,2	15,4	15,5	10,6	26,8
MALATE	0,055	0,069	0,130	0,228	0,212	0,241	0,271	0,288
ADENOSINE-5'-MONOPHOSPHATE	0,125	0,126	0,142	0,219	0,156	0,159	0,132	0,267
ADENOSINE-5'-DIPHOSPHATE	0,489	0,528	0,648	0,783	0,708	0,682	0,418	0,701
ADENOSINE-5'-TRIPHOSPHATE	4,37	4,32	4,86	5,07	5,30	5,53	2,82	3,23
CREATINE PHOSPHATE	10,30	8,98	9,12	9,32	9,95	10,12	3,45	2,32

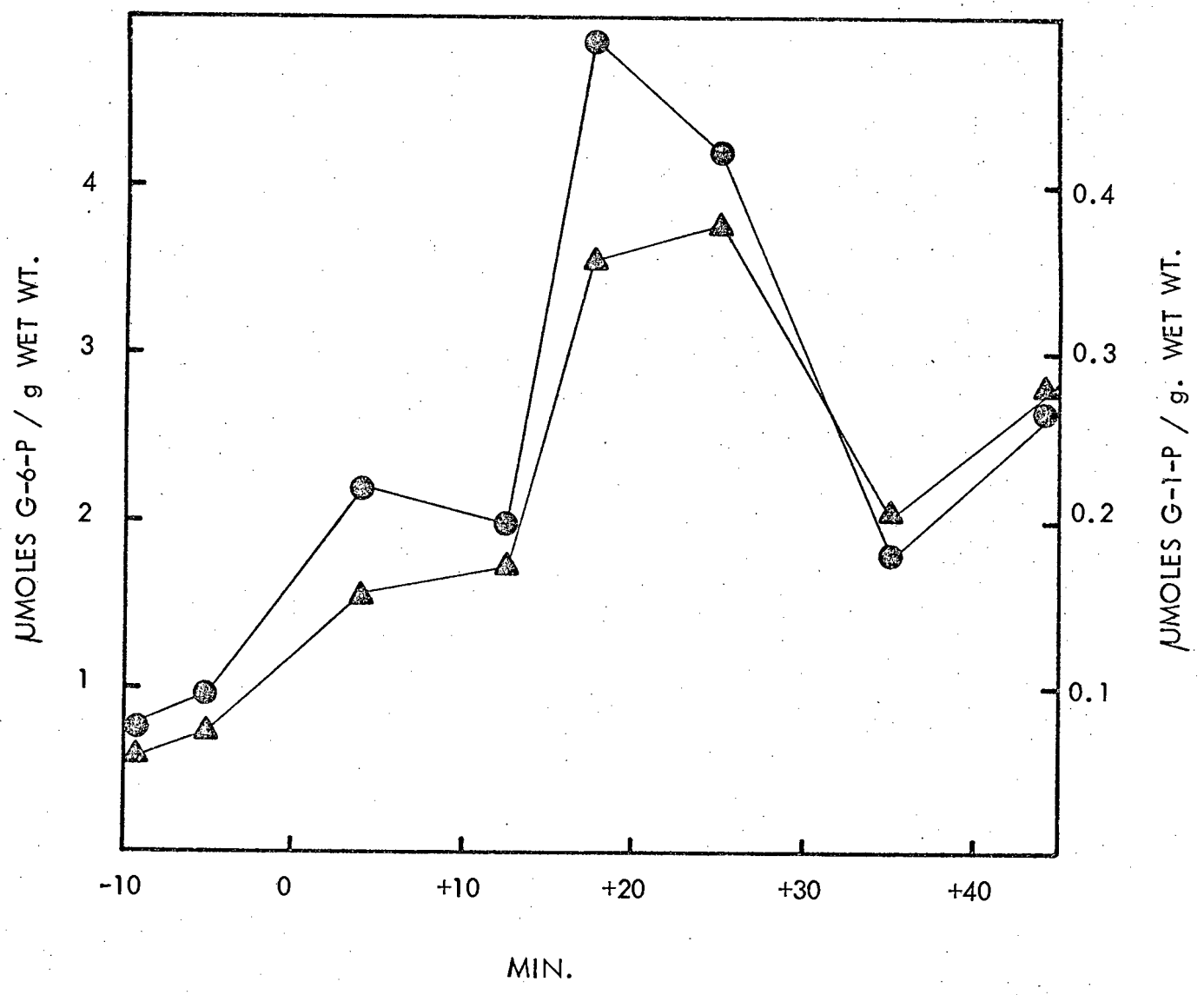


FIG. 15a. PIG 139. MUSCLE G-1-P (●——●) AND G-6-P (▲——▲). HALOTHANE ADMINISTERED AT 0 MIN. AND SUCCINYL CHOLINE AT 10 MIN.

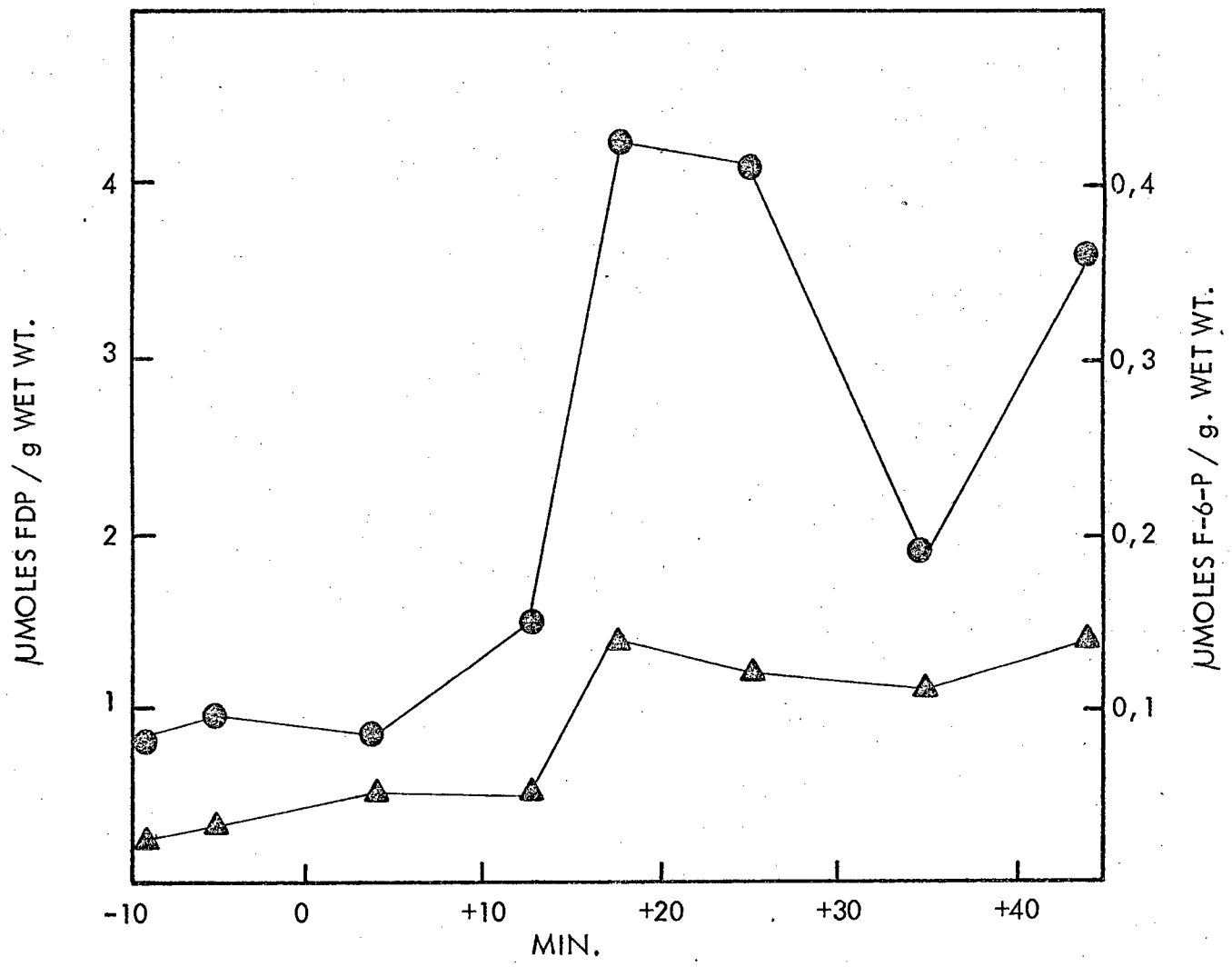


FIG. 15b. PIG 139. MUSCLE F-6-P (●——●) AND FDP (▲——▲). EXPERIMENTAL DETAILS AS IN FIG. 15a.

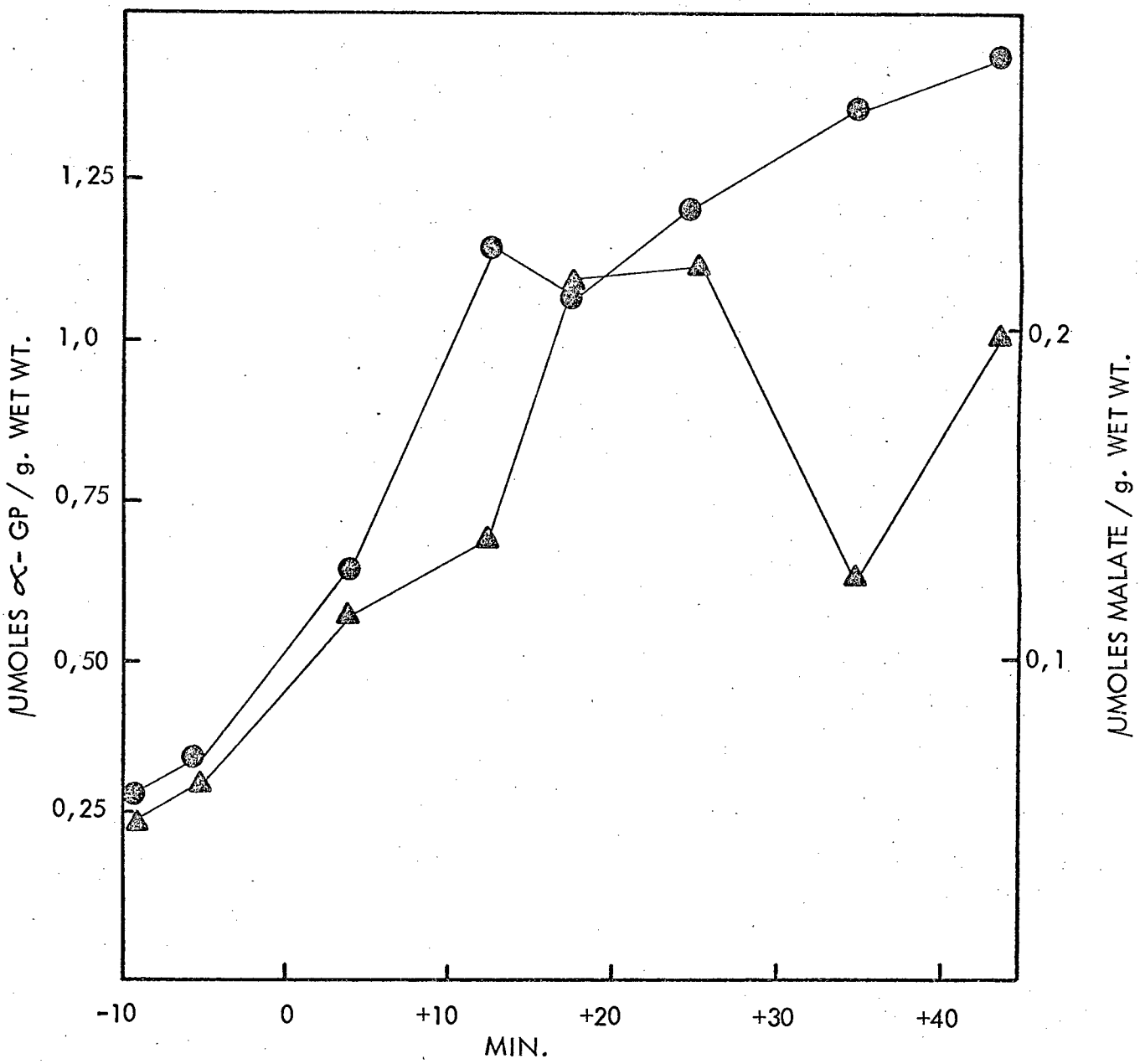


FIG. 15c. PIG 139. MUSCLE ∝-GP (▲——▲) AND MALATE (●——●). EXPERIMENTAL DETAILS AS IN FIG. 15a.

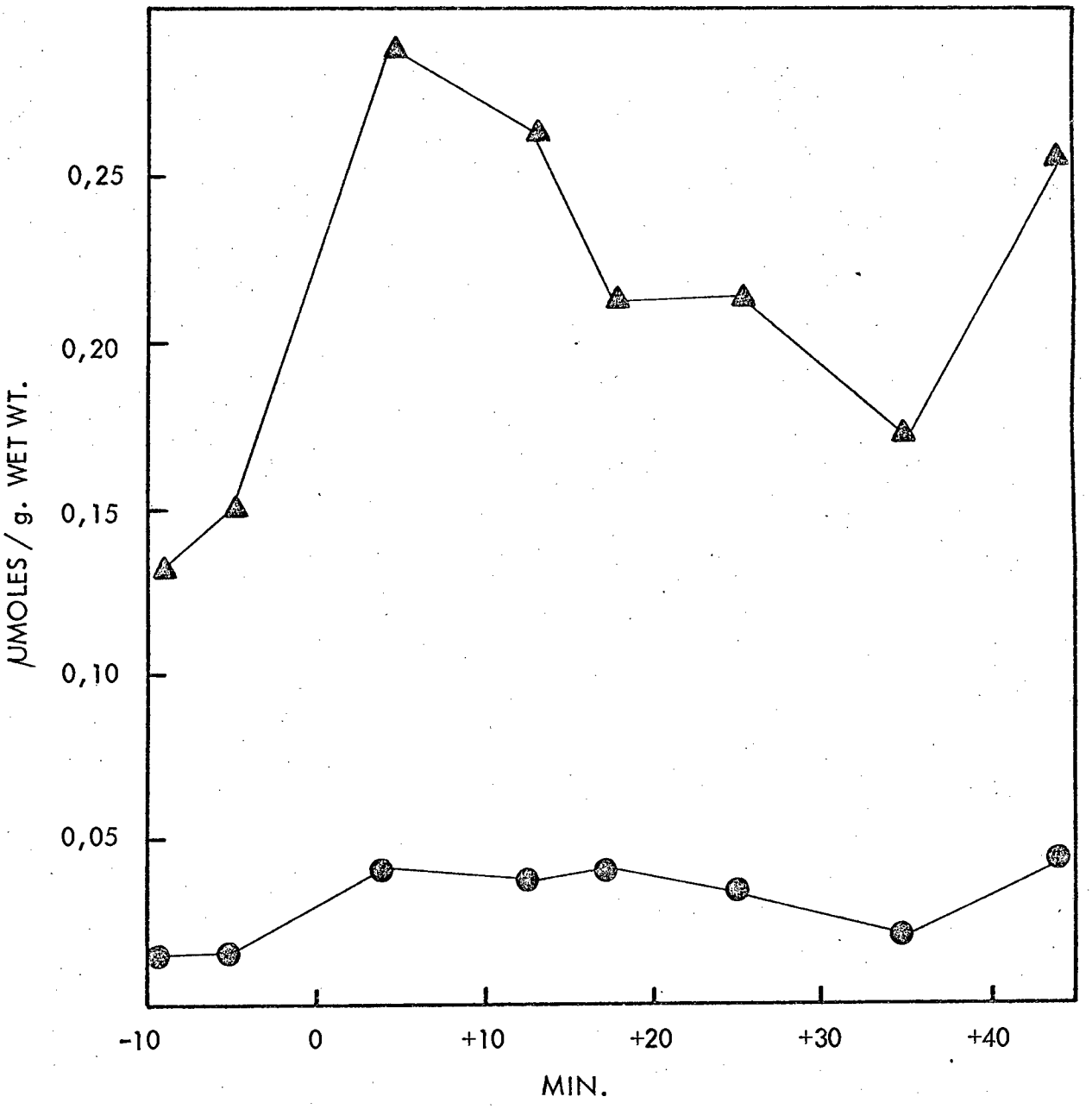


FIG. 15d. PIG 139. MUSCLE 3-PG (▲——▲) AND 2-PG (●——●). EXPERIMENTAL DETAILS AS IN FIG. 15a.

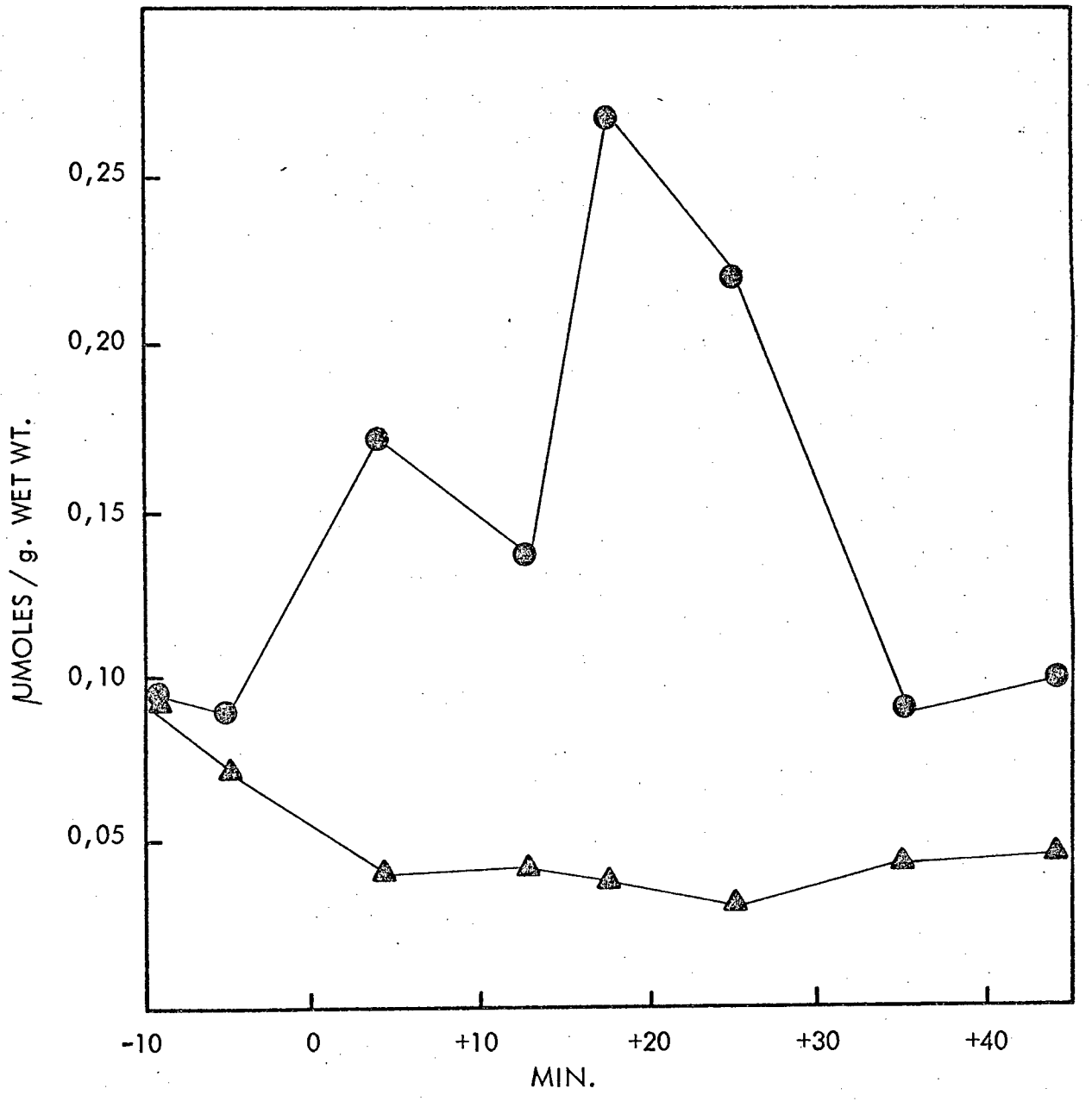


FIG. 15e. PIG 139. MUSCLE PEP (▲——▲) AND PYRUVATE (●——●). EXPERIMENTAL DETAILS AS IN FIG. 15a.

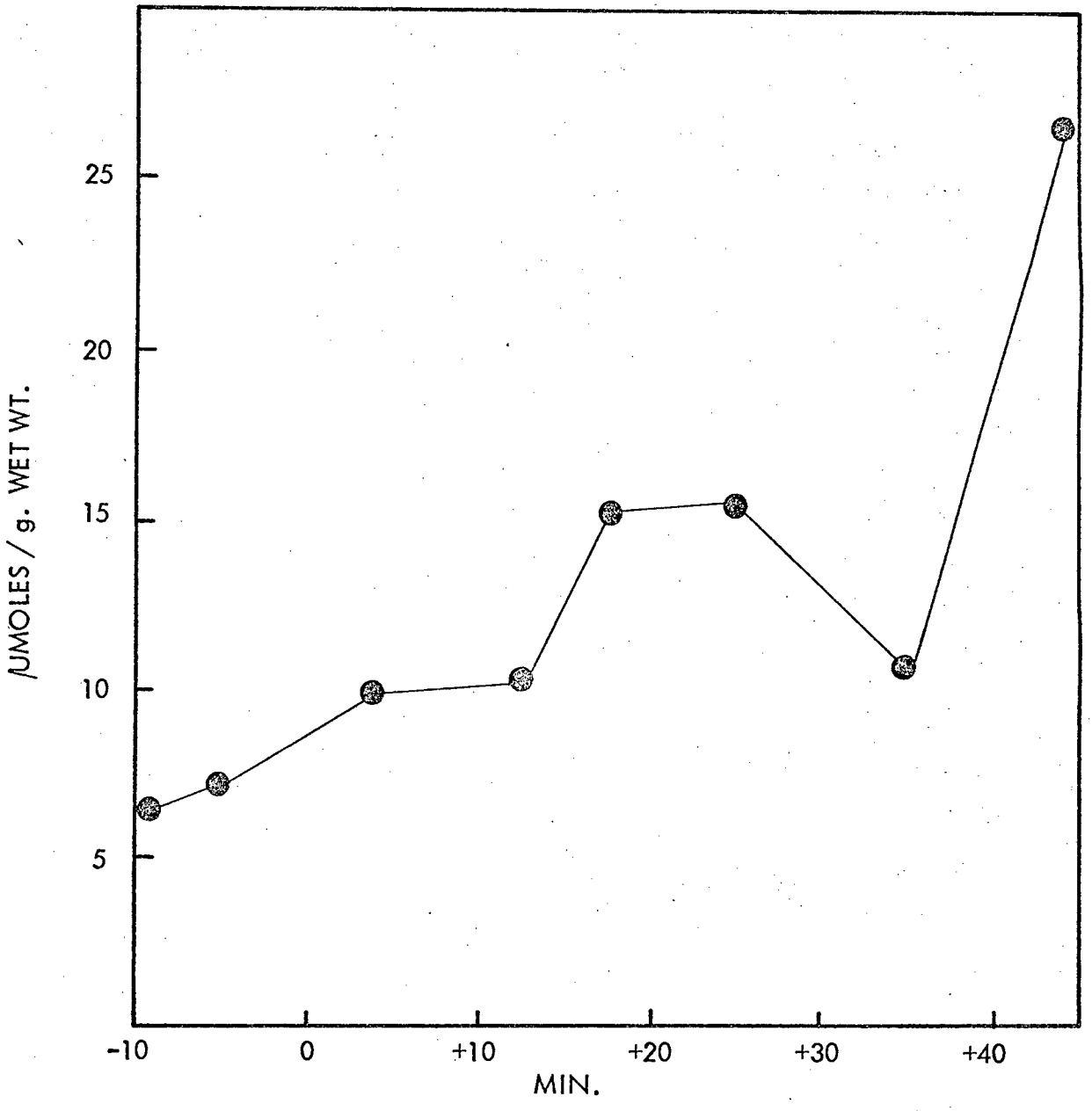


FIG. 15 f. PIG 139. MUSCLE LACTATE. EXPERIMENTAL DETAILS AS IN FIG. 15a.

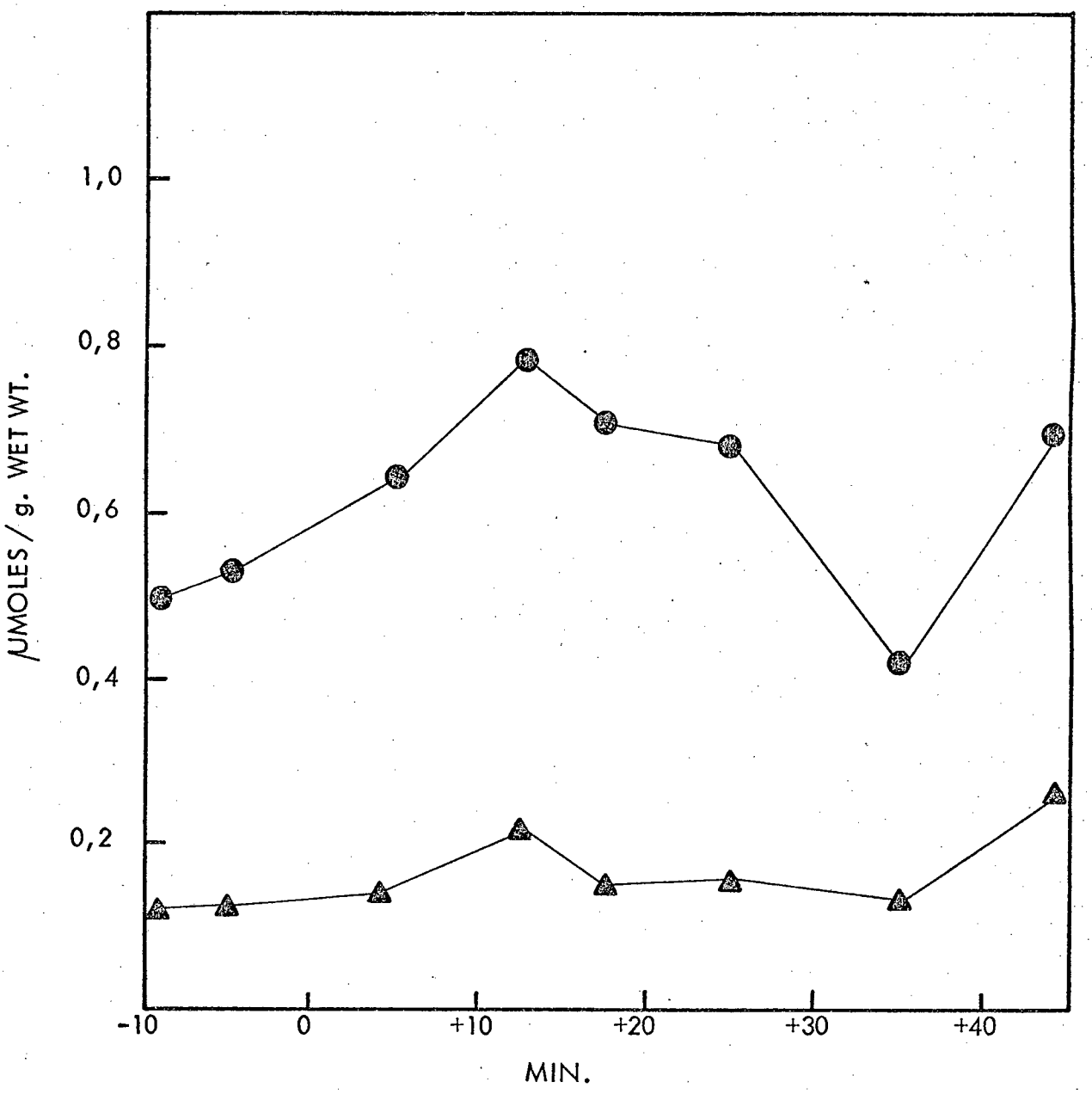


FIG. 15g. PIG 139. MUSCLE AMP (▲——▲) AND ADP (●——●). EXPERIMENTAL DETAILS AS IN FIG. 15a.

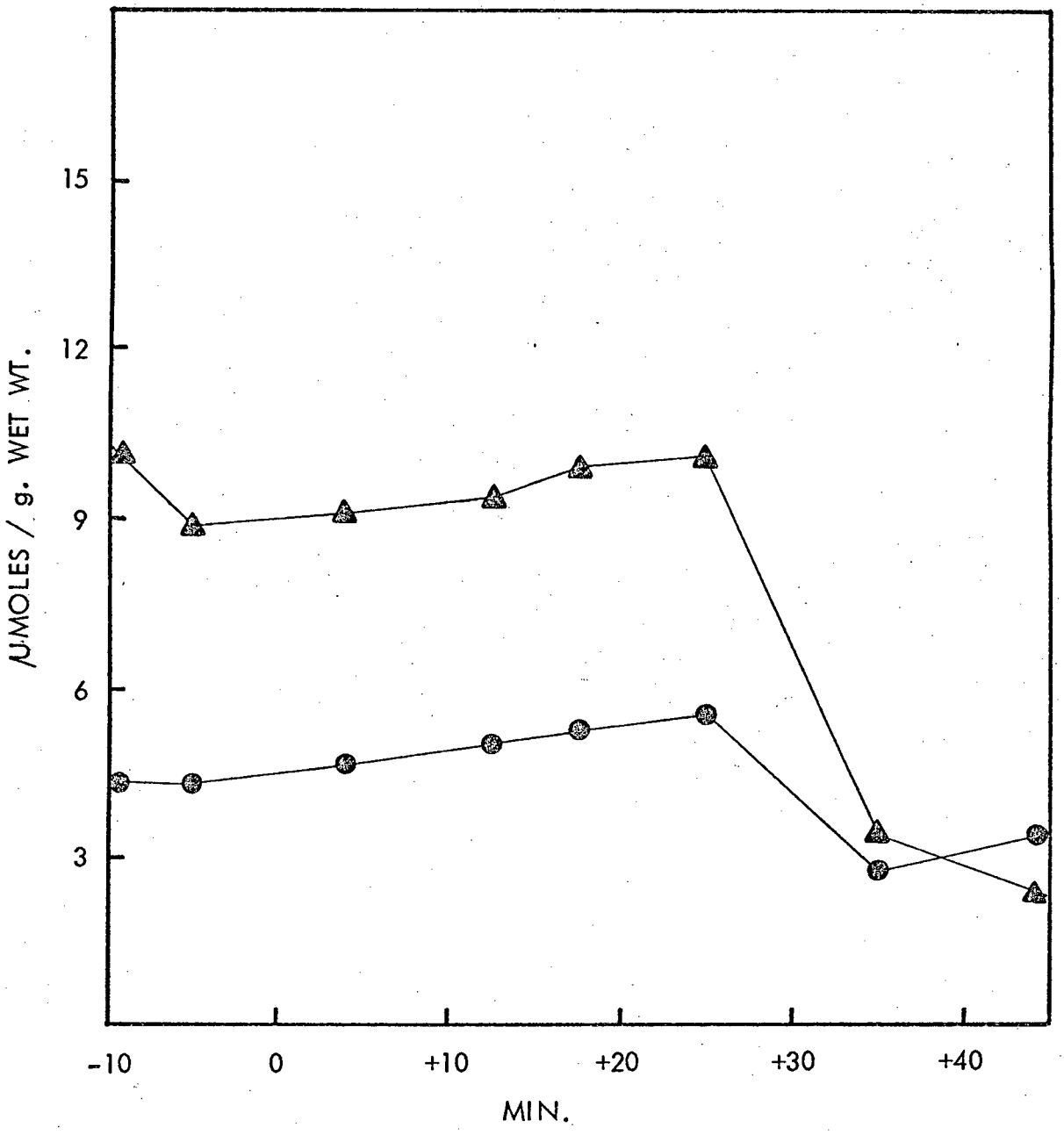


FIG. 15h. , PIG 139. MUSCLE ATP (●——●) AND CP (▲——▲).
EXPERIMENTAL DETAILS AS IN FIG. 15a.

TABLE IV

GLYCOLYTIC INTERMEDIATES AND COFACTORS IN SKELETAL MUSCLE (PIG 137)

(μMOLES/G. WET WT.)

MIN	-5	+2	+5	+15	+20	+25
GLUCOSE-1-PHOSPHATE	0,047	0,037	0,024	0,084	0,068	0,404
GLUCOSE-6-PHOSPHATE	0,956	0,656	0,529	0,831	0,627	7,98
FRUCTOSE-6-PHOSPHATE	0,102	0,094	0,073	0,071	0,068	0,933
FRUCTOSE-1,6-DIPHOSPHATE	0,450	0,887	1,14	1,42	1,28	1,87
∝-GLYCEROL PHOSPHATE	0,411	0,571	0,605	0,650	0,903	1,27
3-PHOSPHOGLYCERATE	0,134	0,155	0,181	0,191	0,173	0,121
2-PHOSPHOGLYCERATE	0,047	0,058	0,055	0,045	0,050	0,050
PHOSPHOENOLPYRUVATE	0,063	0,079	0,079	0,076	0,087	0,084
PYRUVATE	0,228	0,168	0,168	0,124	0,121	0,393
LACTATE	11,9	10,2	13,3	13,5	15,9	25,6
MALATE	0,131	0,126	0,136	0,131	0,155	0,280
ADENOSINE-5'-MONOPHOSPHATE	0,228	0,241	0,265	0,236	0,283	0,283
ADENOSINE-5'-DIPHOSPHATE	0,604	0,668	0,747	0,755	0,750	0,923
ADENOSINE-5'-TRIPHOSPHATE	5,41	7,38	7,35	6,90	6,37	5,89
CREATINE PHOSPHATE	5,83	4,99	5,30	6,68	4,73	3,18

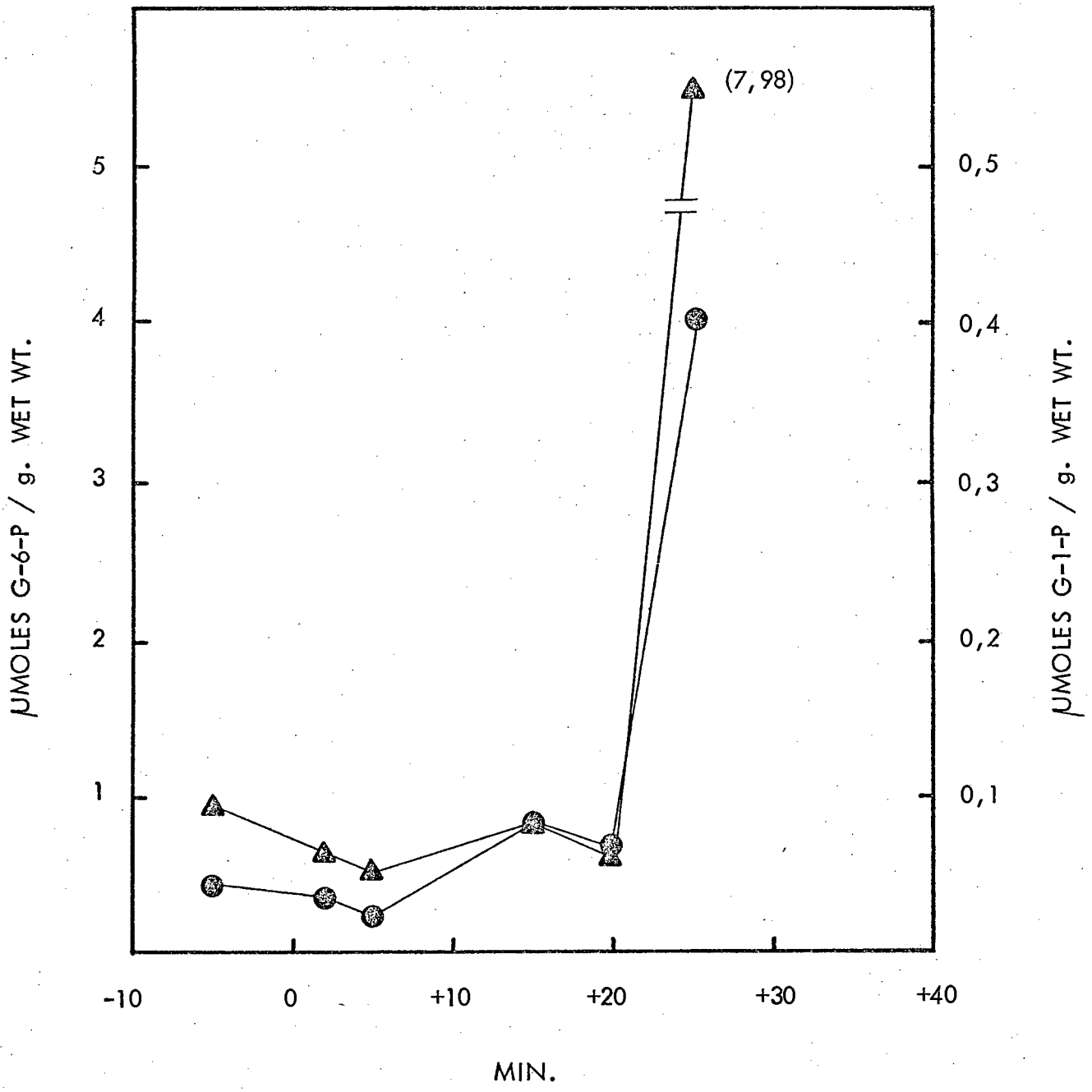


FIG. 16a. PIG 137. MUSCLE G-1-P (●——●) AND G-6-P (▲——▲). HALOTHANE ADMINISTERED AT 0 MIN. AND SUCCINYL CHOLINE AT 14 MIN.

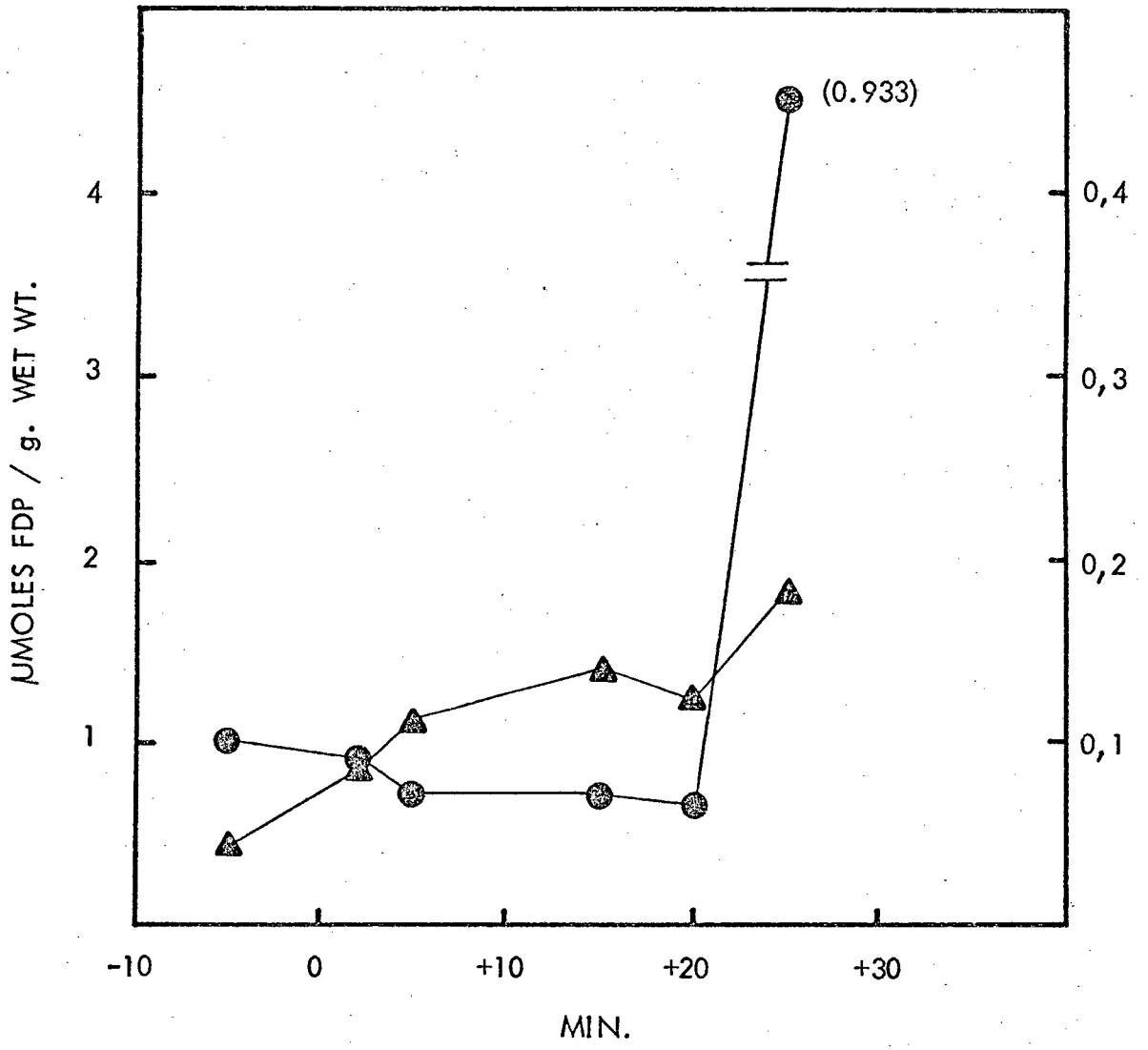


FIG. 16b. PIG 137. MUSCLE F-6-P (●——●) AND FDP (▲——▲). EXPERIMENTAL DETAILS AS IN FIG. 16a.

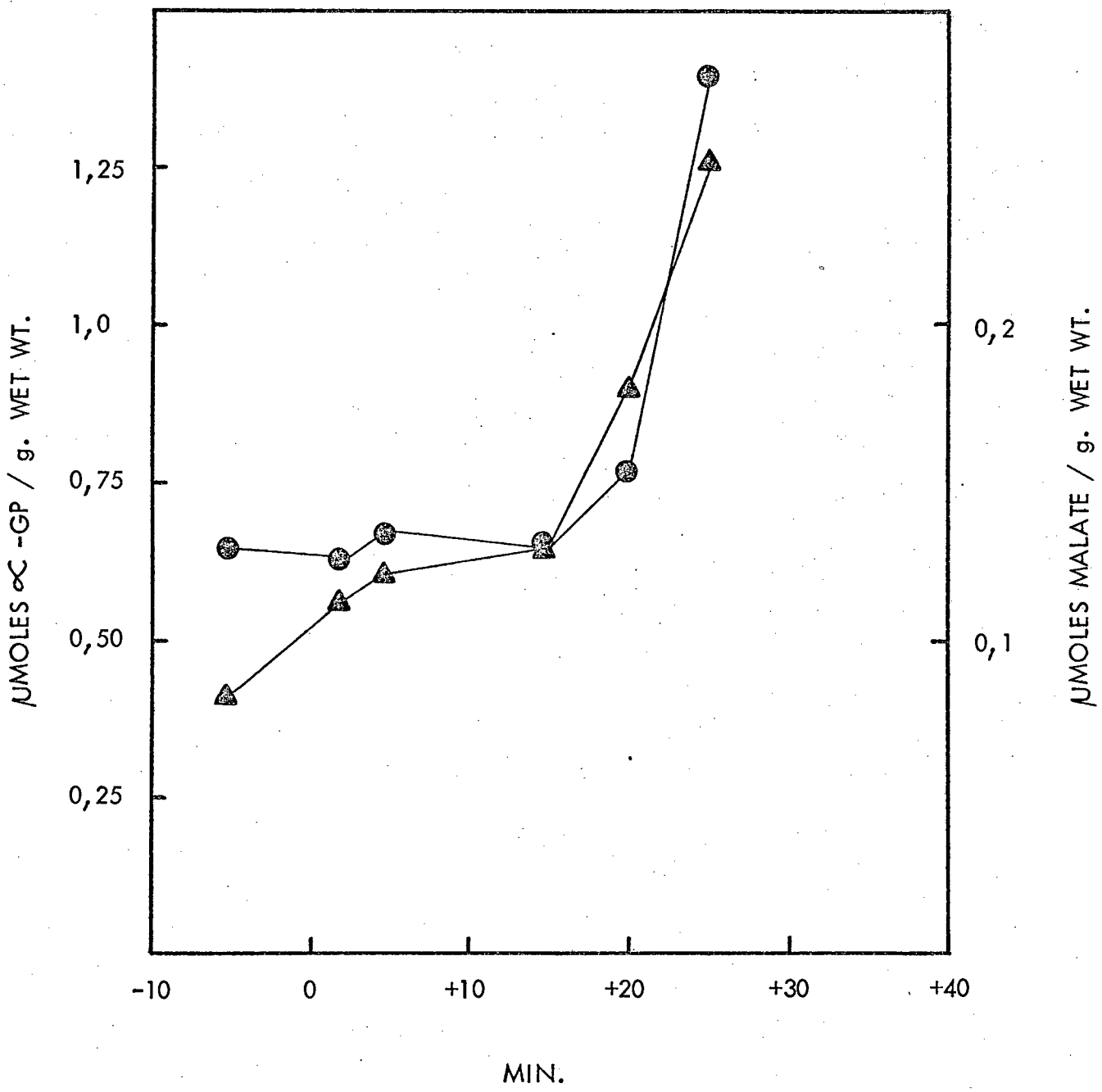


FIG. 16c. PIG 137. MUSCLE ∝-GP (▲——▲) AND MALATE (●——●). EXPERIMENTAL DETAILS AS IN FIG. 16a.

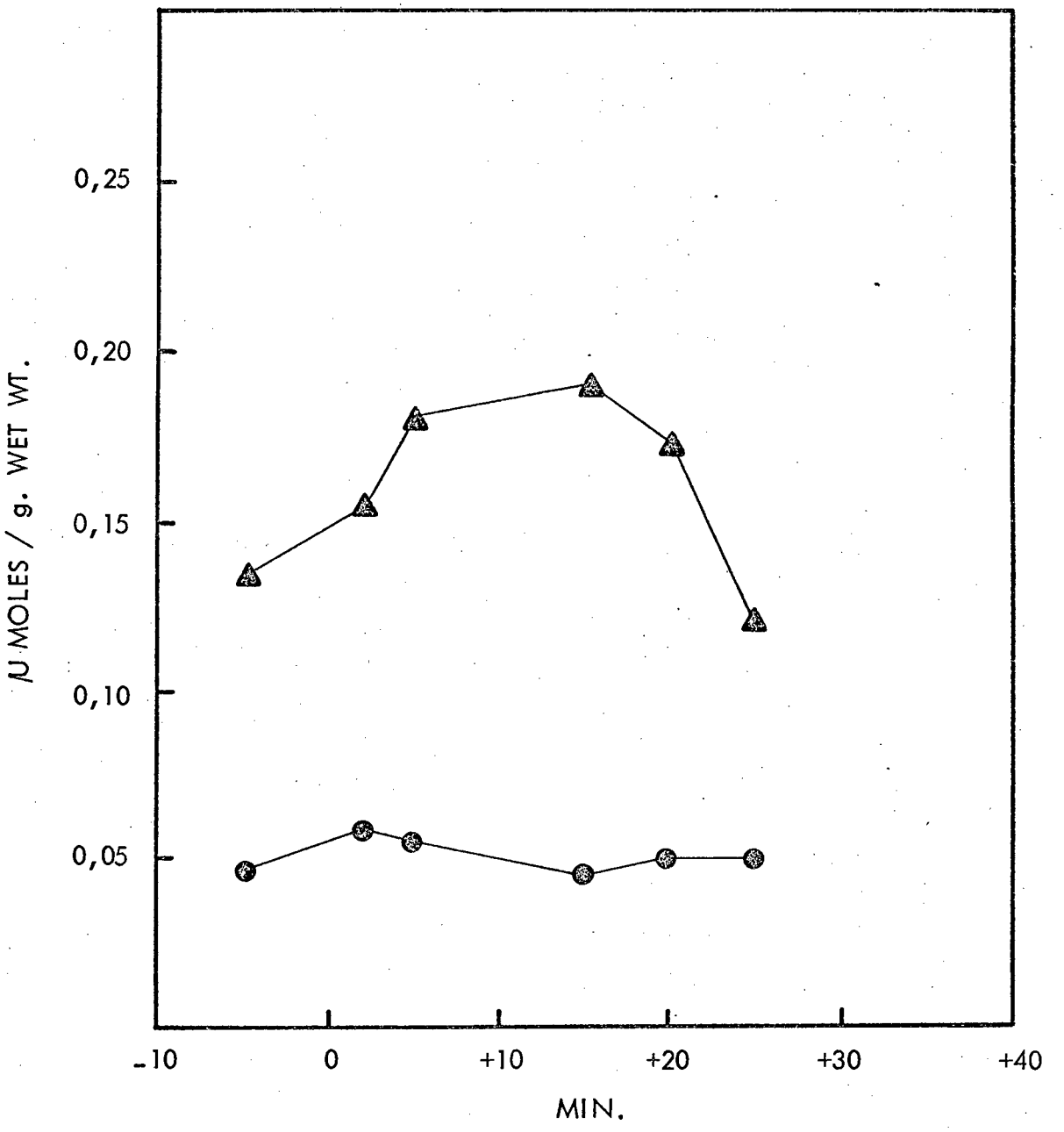


FIG. 16d. PIG 137. MUSCLE 3-PG (▲——▲) AND 2-PG (●——●). EXPERIMENTAL DETAILS AS IN FIG. 16a.

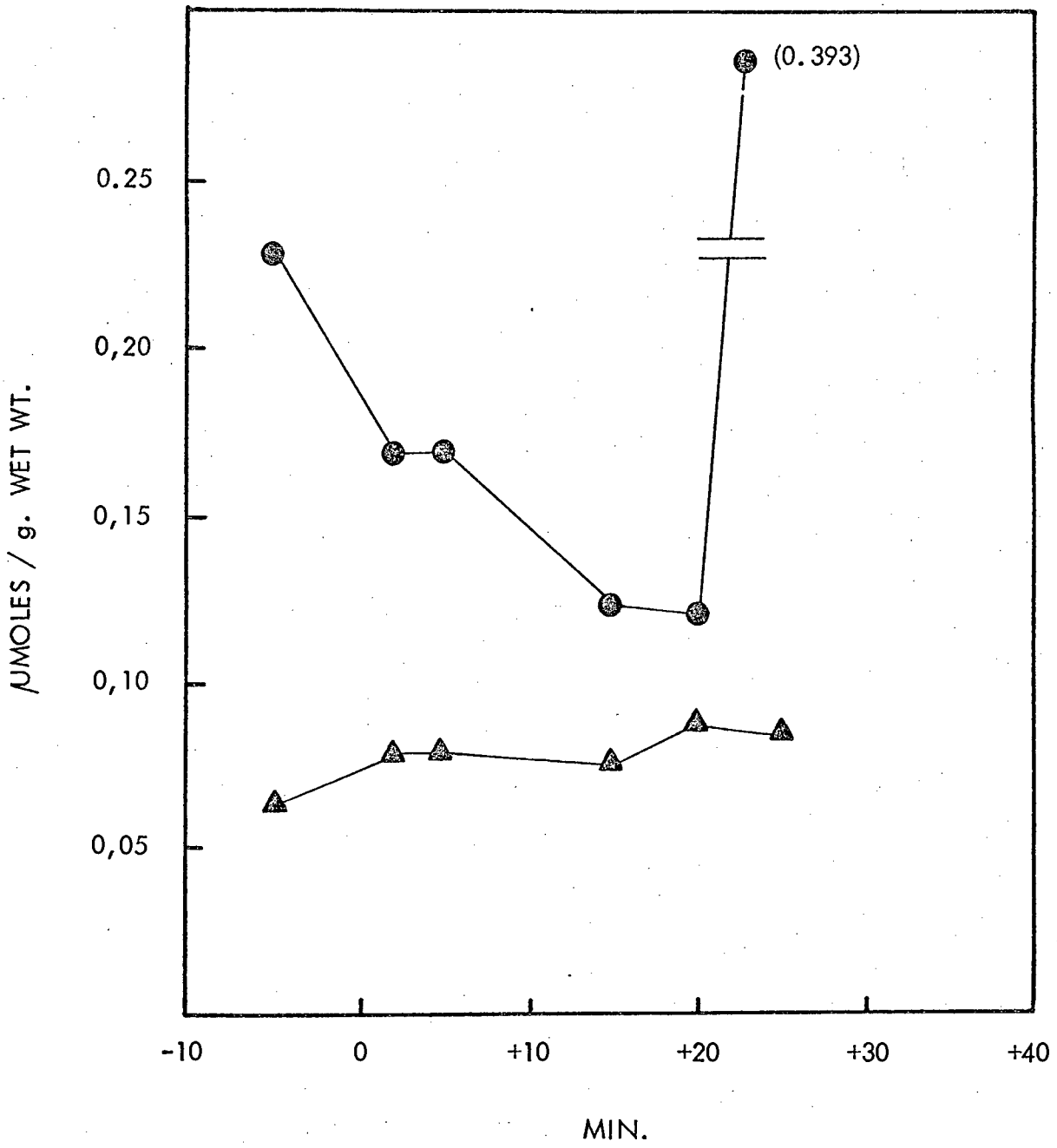


FIG. 16e. PIG 137. MUSCLE PEP (▲——▲) AND PYRUVATE (●——●). EXPERIMENTAL DETAILS AS IN FIG. 16a.

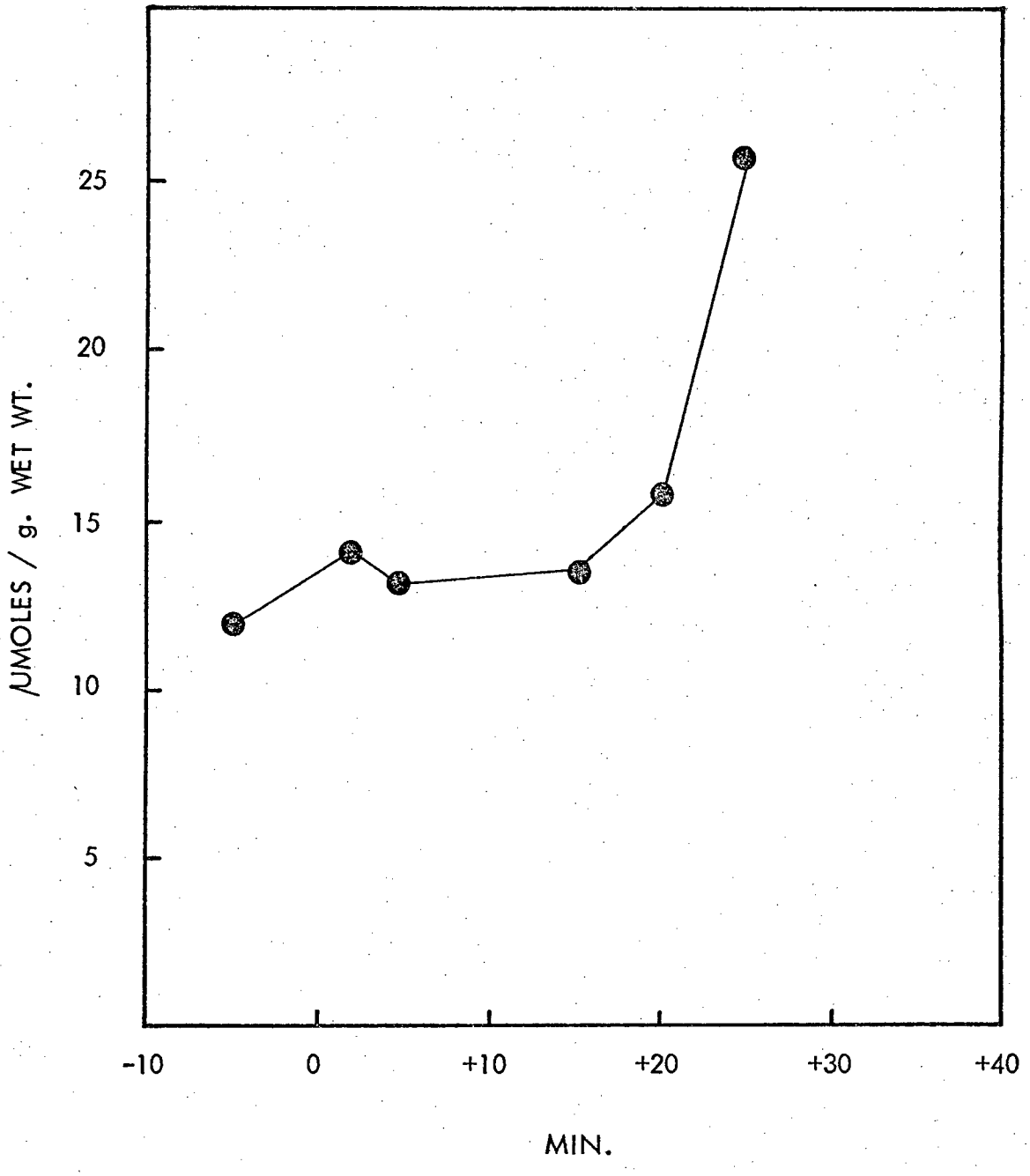


FIG. 16f. PIG 137. MUSCLE LACTATE. EXPERIMENTAL DETAILS AS IN FIG. 16a.

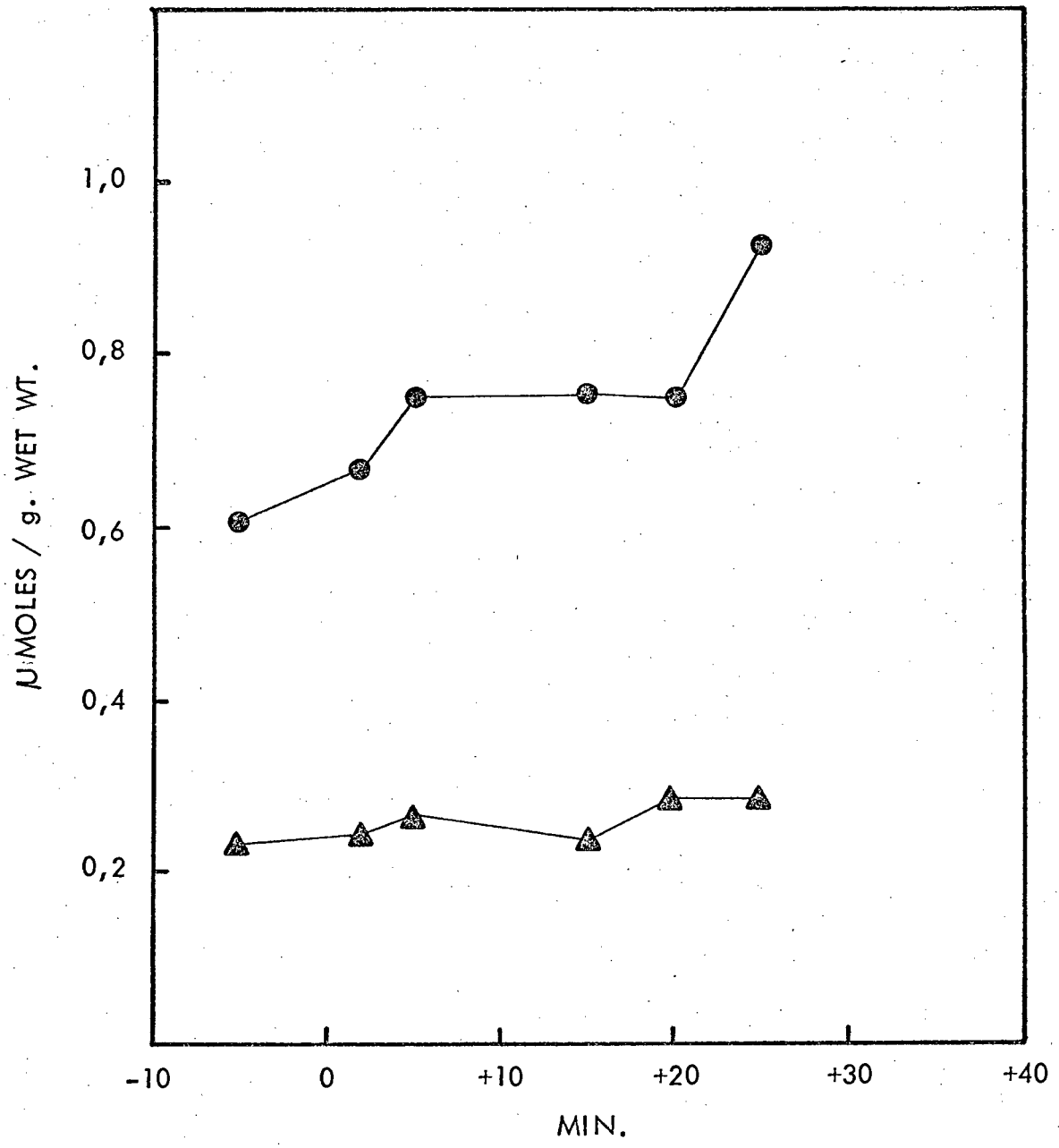


FIG. 16g. PIG 137. MUSCLE AMP (▲——▲) AND ADP (●——●). EXPERIMENTAL DETAILS AS IN FIG. 16a.

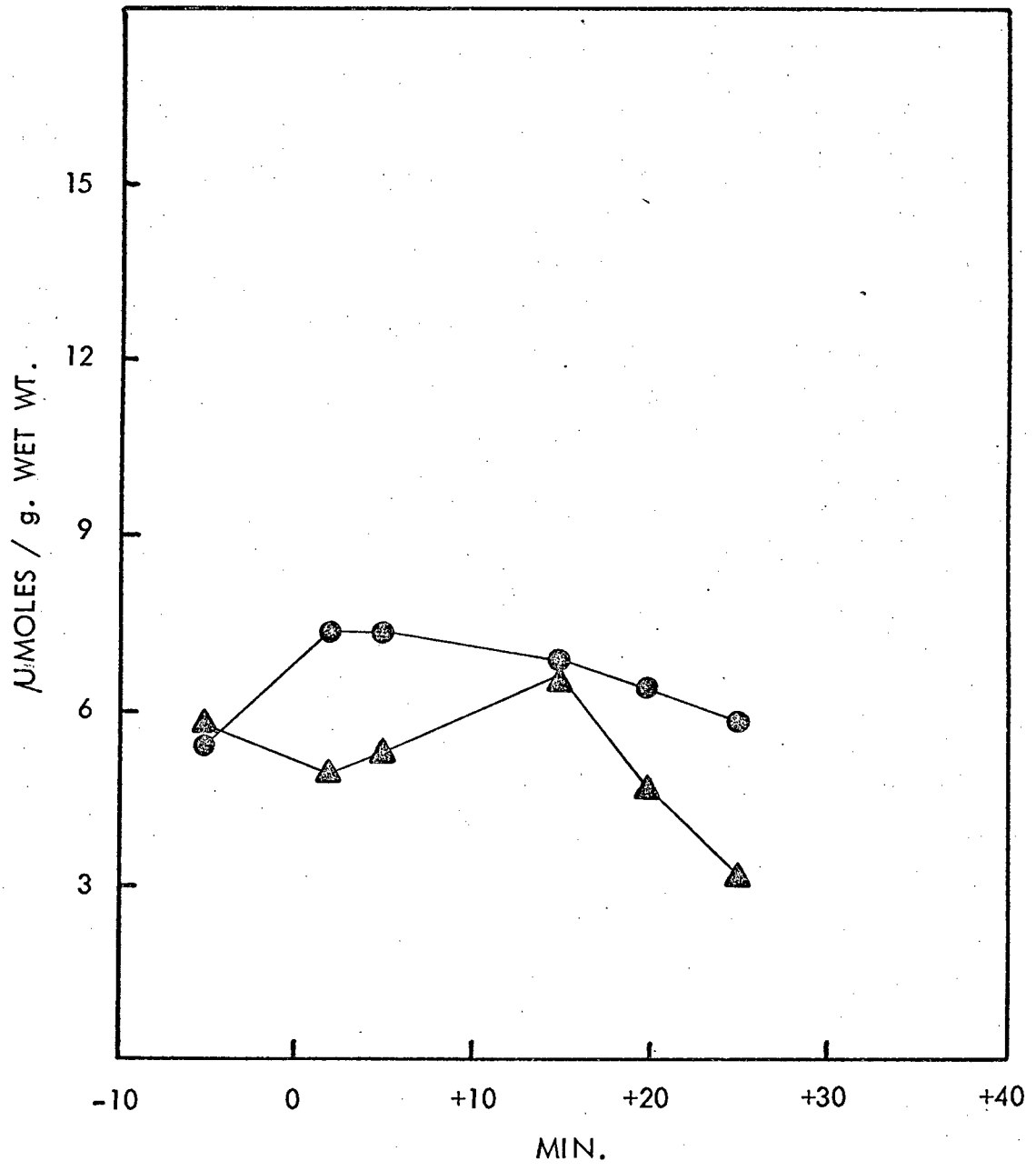


FIG. 16h. PIG 137. MUSCLE ATP (●——●) AND CP (▲——▲).
EXPERIMENTAL DETAILS AS IN FIG. 16a.

TABLE V

MASS/ACTION RELATIONSHIP OF GLYCOLYTIC REACTIONS (PIG 22B)

MIN	-10	+1	+7	+10	+13	+14	+19	+24	+30	+38	Equilibrium constants *
$\frac{[3\text{-PG}]}{[2\text{-PG}]}$	3,35	3,57	3,06	2,86	3,70	3,45	1,86	2,33	3,44	2,60	6,5
$\frac{[2\text{-PG}]}{[PEP][H_2O]}$	0,712	0,72	0,91	0,93	0,84	0,91	1,48	1,14	0,96	1,23	$1,6 \times 10^{-1}$
$\frac{[PEP][ADP]}{[PYR][ATP]}\ \frac{4}{5}$	0,051	0,069	0,073	0,068	0,079	0,047	0,047	0,096	0,091	0,080	$8,7 \times 10^{-4}$

* (1) WILLIAMSON, (1965)

TABLE VI

MASS/ACTION RELATIONSHIP OF GLYCOLYTIC REACTIONS (PIG 139)

MIN	-10	-5	+4	+12½	+17½	+25	+35	+45	Equilibrium constants *
$\frac{[G\text{-}1\text{-}P]}{[G\text{-}6\text{-}P]}$	0,126	0,130	0,141	0,116	0,136	0,112	0,089	0,095	$5,8 \times 10^{-2}$
$\frac{[G\text{-}6\text{-}P]}{[F\text{-}6\text{-}P]}$	7,21	7,67	17,9	11,3	8,33	9,06	10,3	7,66	3,85
$\frac{[F\text{-}6\text{-}P]\,[ATP]}{[FDP]\,[ADP]}\,\frac{4}{5}$	2,61	1,80	0,96	1,49	1,78	2,15	0,92	0,95	$8,3 \times 10^{-4}$
$\frac{[3\text{-}PG]}{[2\text{-}PG]}$	8,60	9,43	6,88	7,13	5,04	6,29	8.19	5,75	6,5
$\frac{[2\text{-}PG]}{[PEP]\,[H_2O]}$	0,163	0,225	1,05	0,88	1,14	1,10	0,488	1,00	$1,6 \times 10^{-1}$
$\frac{[PEP]\,[ADP]}{[PYR]\,[ATP]}\,\frac{4}{5}$	0,085	0,077	0,023	0,036	0,014	0,013	0,054	0,078	$8,7 \times 10^{-4}$

* (1) WILLIAMSON, (1965)

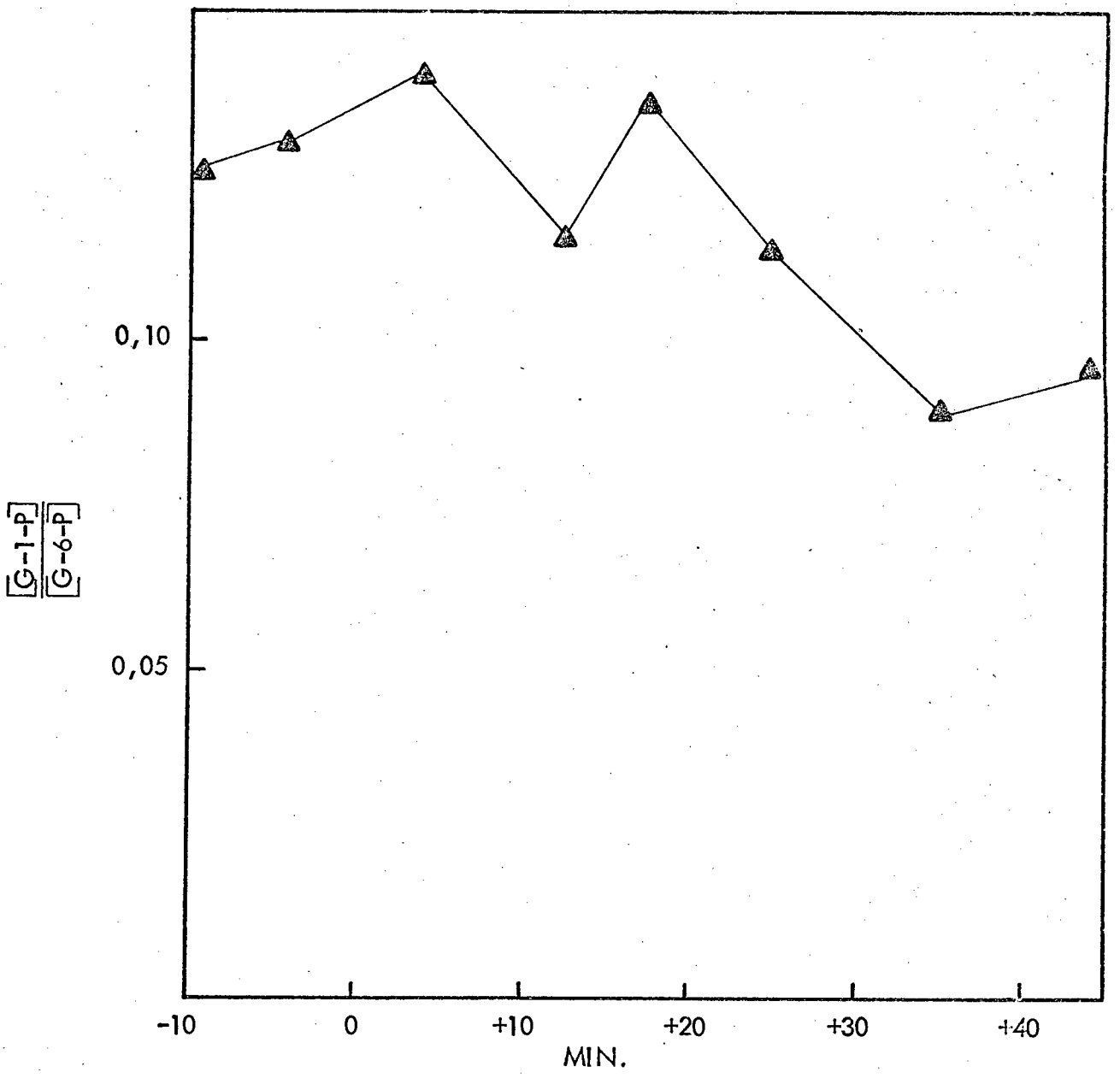


FIG. 17a. PIG 139. THE MASS/ACTION RELATIONSHIP OF THE PHOSPHOGLUCOMUTASE REACTION CALCULATED FROM SKELETAL MUSCLE CONTENT OF G-1-P AND G-6-P. EXPERIMENTAL DETAILS AS IN FIG. 15a.

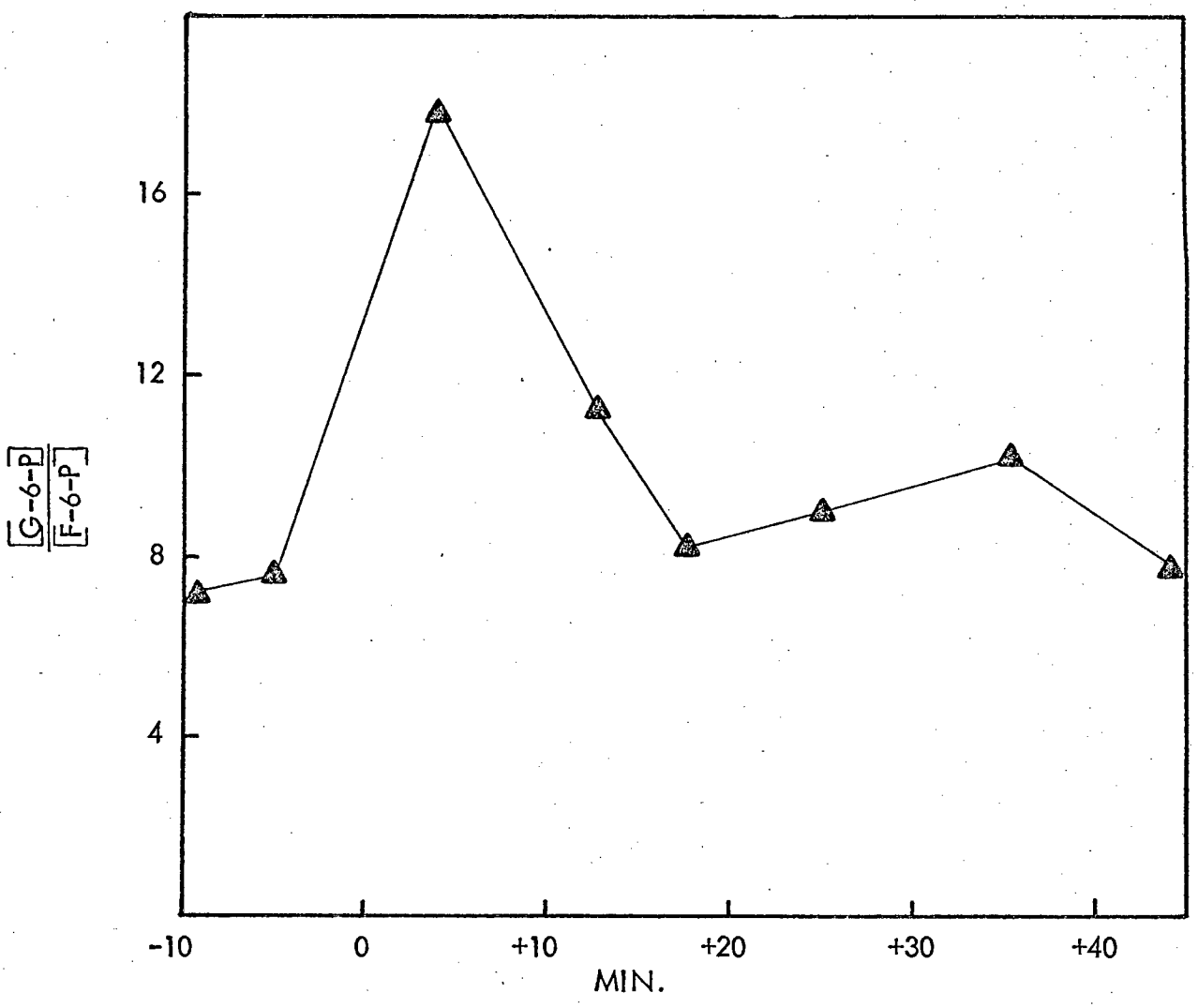


FIG. 17b. PIG 139. THE MASS/ACTION RELATIONSHIP OF THE PHOSPHOGLUCOSE ISOMERASE REACTION CALCULATED FROM SKELETAL MUSCLE CONTENT OF G-6-P AND F-6-P. EXPERIMENTAL DETAILS AS IN FIG. 15a.

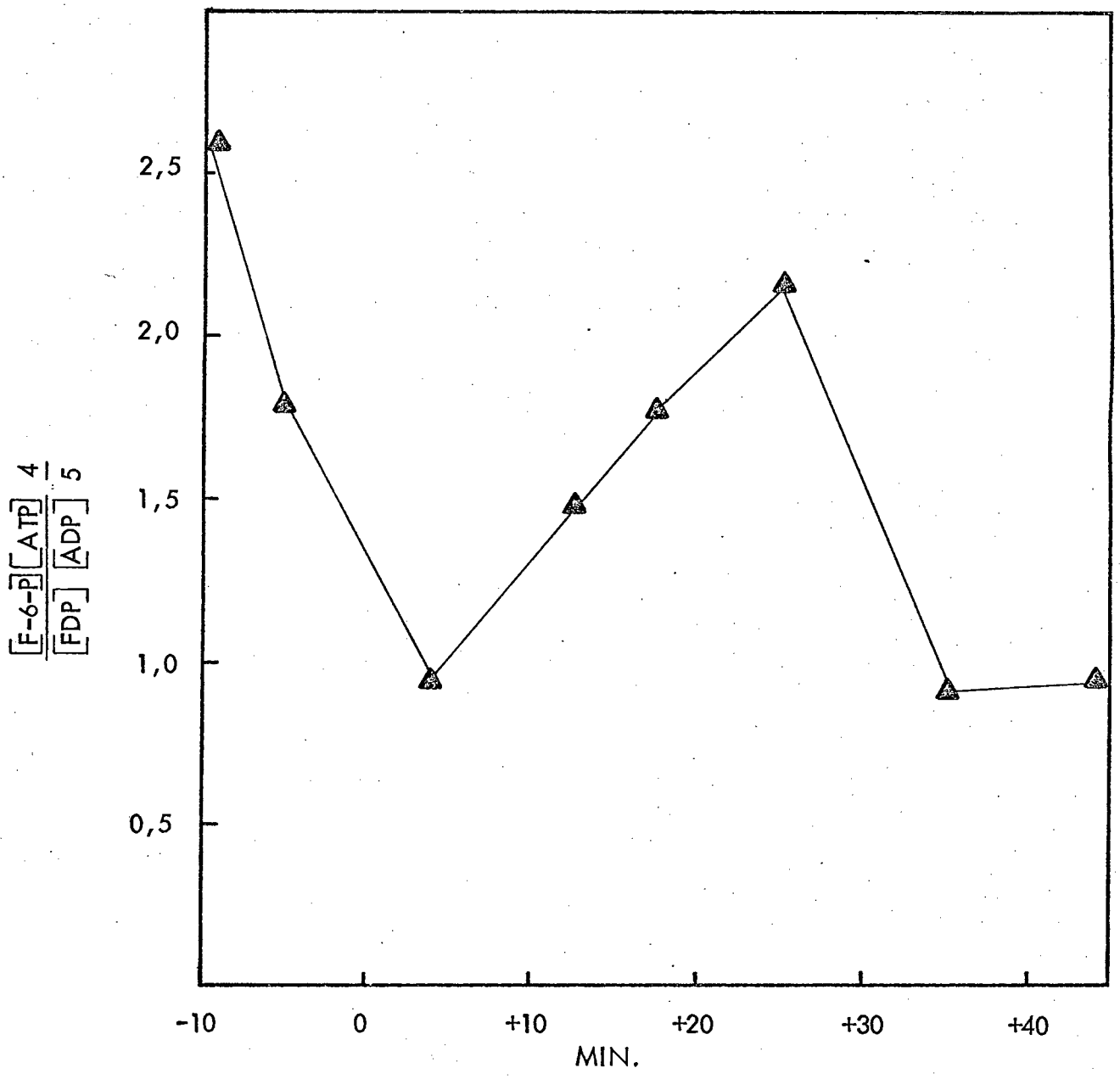


FIG. 17c. PIG 139. THE MASS/ACTION RELATIONSHIP OF THE PHOSPHOFRUCTOKINASE REACTION CALCULATED FROM SKELETAL MUSCLE CONTENT OF F-6-P AND FDP. EXPERIMENTAL DETAILS AS IN FIG. 15a.

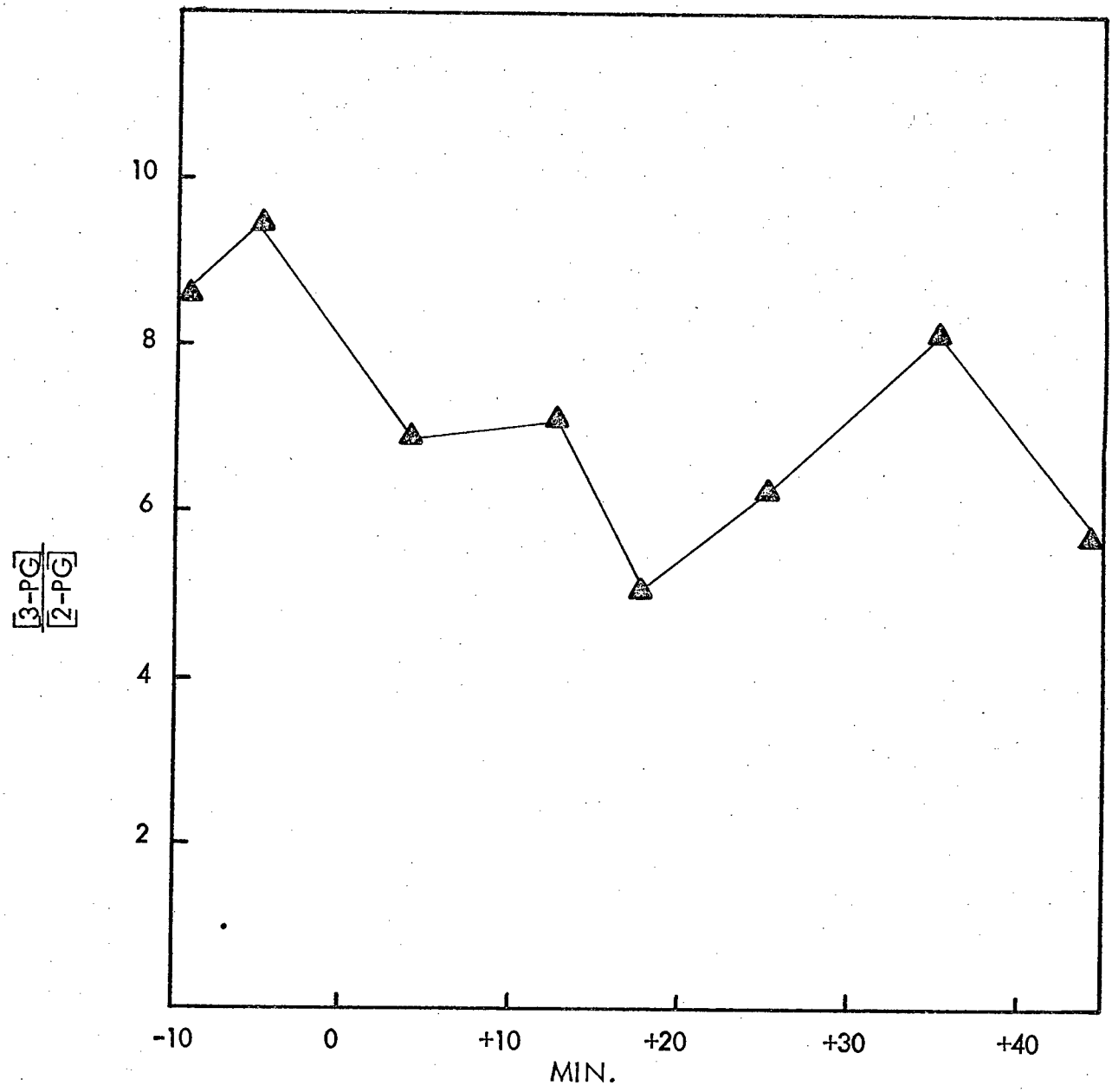


FIG. 17d. PIG 139. THE MASS/ACTION RELATIONSHIP OF THE PHOSPHOGLYCERATE MUTASE REACTION CALCULATED FROM SKELETAL MUSCLE CONTENT OF 3-PG AND 2-PG. EXPERIMENTAL DETAILS AS IN FIG. 15a.

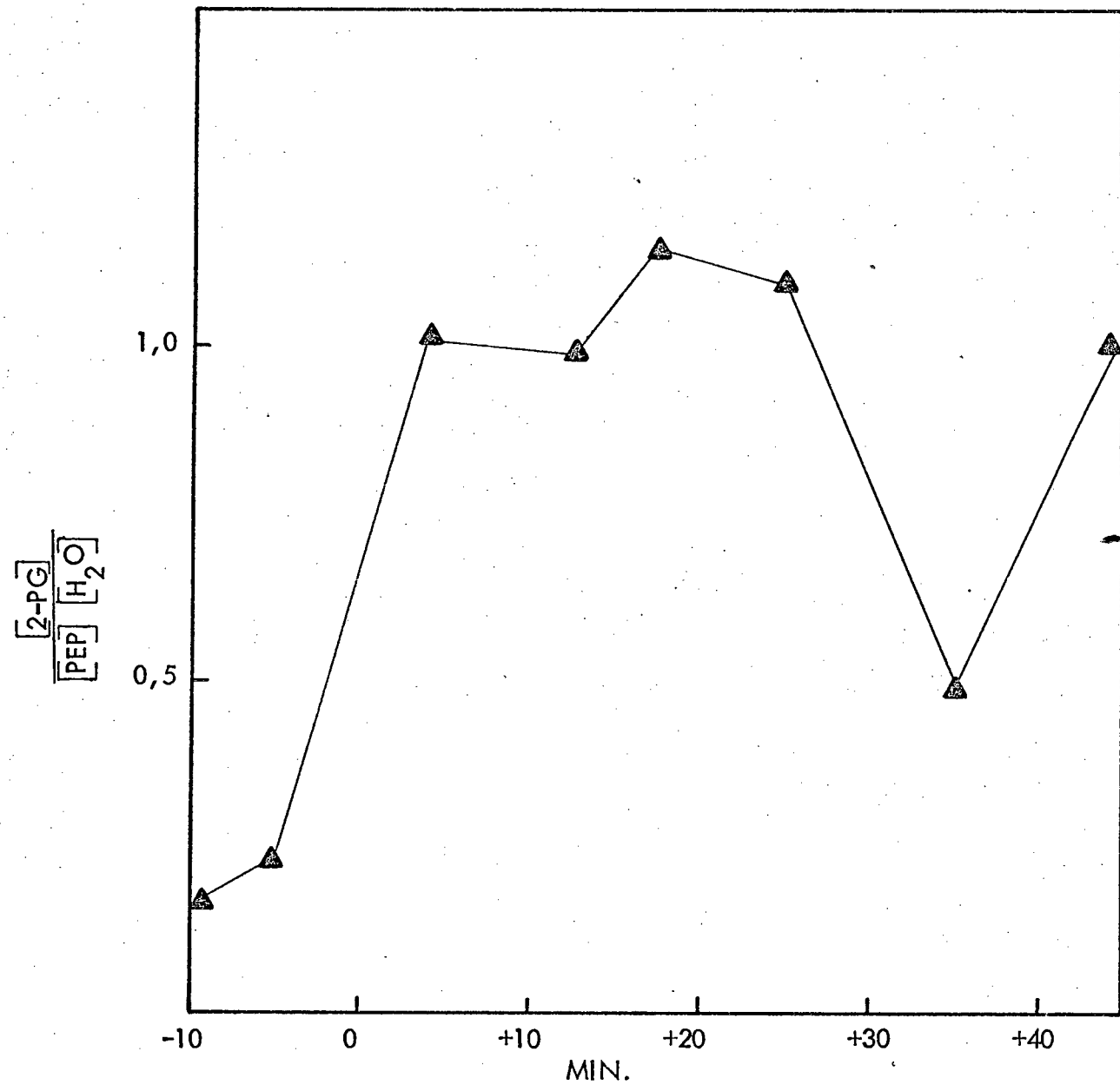


FIG. 17e. PIG 139. THE MASS/ACTION RELATIONSHIP OF THE ENOLASE REACTION CALCULATED FROM SKELETAL MUSCLE CONTENT OF 2-PG AND PEP. EXPERIMENTAL DETAILS AS IN FIG. 15a.

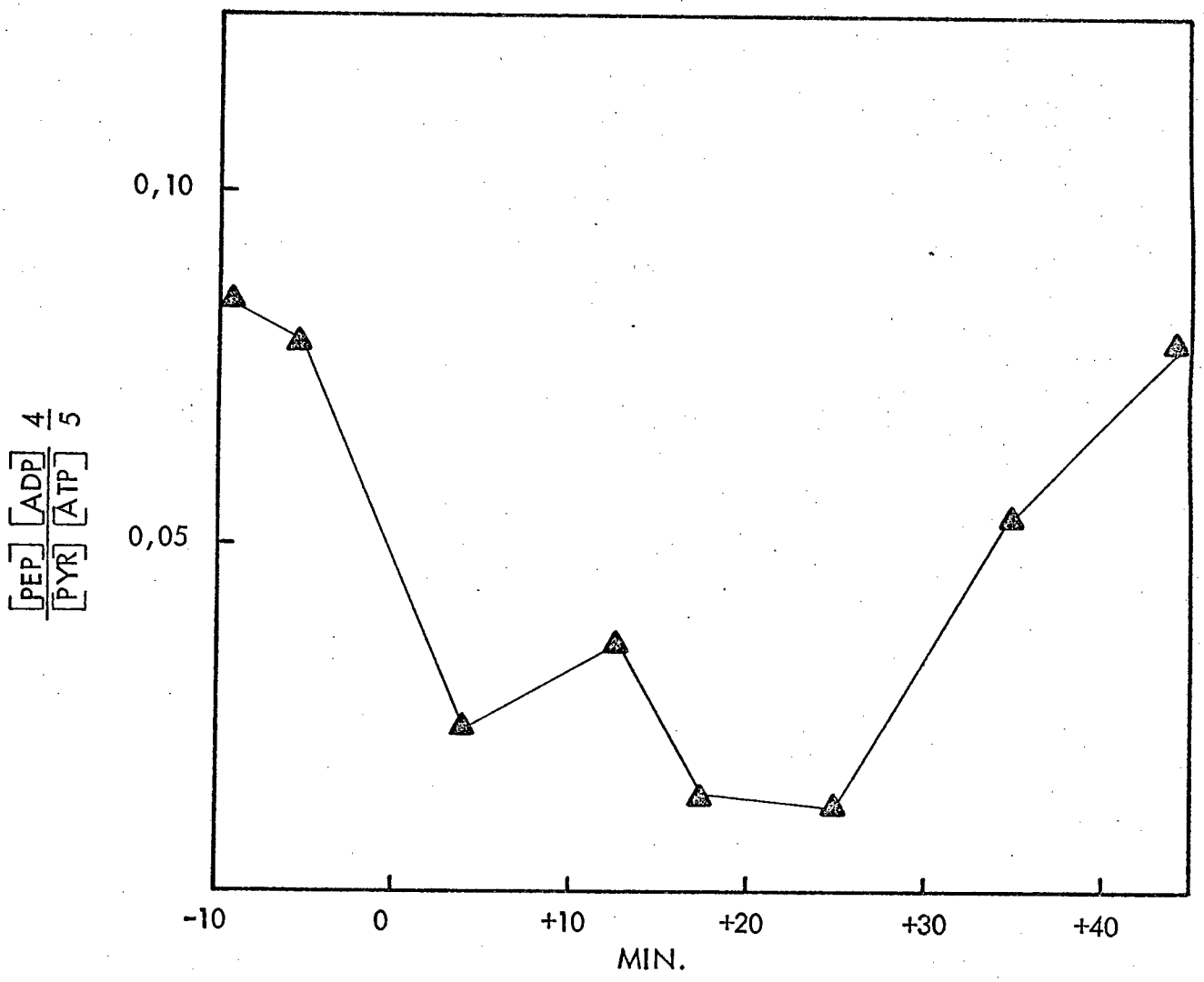


FIG. 17f. PIG 139. THE MASS ACTION RELATIONSHIP OF THE PYRUVATE KINASE REACTION CALCULATED FROM SKELETAL MUSCLE CONTENT OF PEP AND PYRUVATE. EXPERIMENTAL DETAILS AS IN FIG. 15a.

TABLE V II

MASS/ACTION RELATIONSHIP OF GLYCOLYTIC REACTIONS (PIG 137)

MIN.	-5	+2	+5	+15	+20	+25	Equilibrium constants *
$\frac{[G\text{-}1\text{-}P]}{[G\text{-}6\text{-}P]}$	0,049	0,056	0,045	0,101	0,108	0,050	$5,8 \times 10^{-2}$
$\frac{[G\text{-}6\text{-}P]}{[F\text{-}6\text{-}P]}$	9,38	6,98	7,25	11,7	9,22	8,55	3,85
$\frac{[F\text{-}6\text{-}P][ATP]}{[FDP][ADP]}\frac{4}{5}$	1,63	0,936	0,505	0,366	0,361	2,54	$8,3 \times 10^{-4}$
$\frac{[3\text{-}PG]}{[2\text{-}PG]}$	2,85	2,67	3,29	4,24	3,46	2,42	6,5
$\frac{[2\text{-}PG]}{[PEP][H_2O]}$	0,75	0,73	0,70	0,59	0,57	0,60	$1,6 \times 10^{-1}$
$\frac{[PEP][ADP]}{[PYR][ATP]}\frac{4}{5}$	0,024	0,033	0,038	0,053	0,067	0,026	$8,7 \times 10^{-4}$

* (1) WILLIAMSON, (1965)

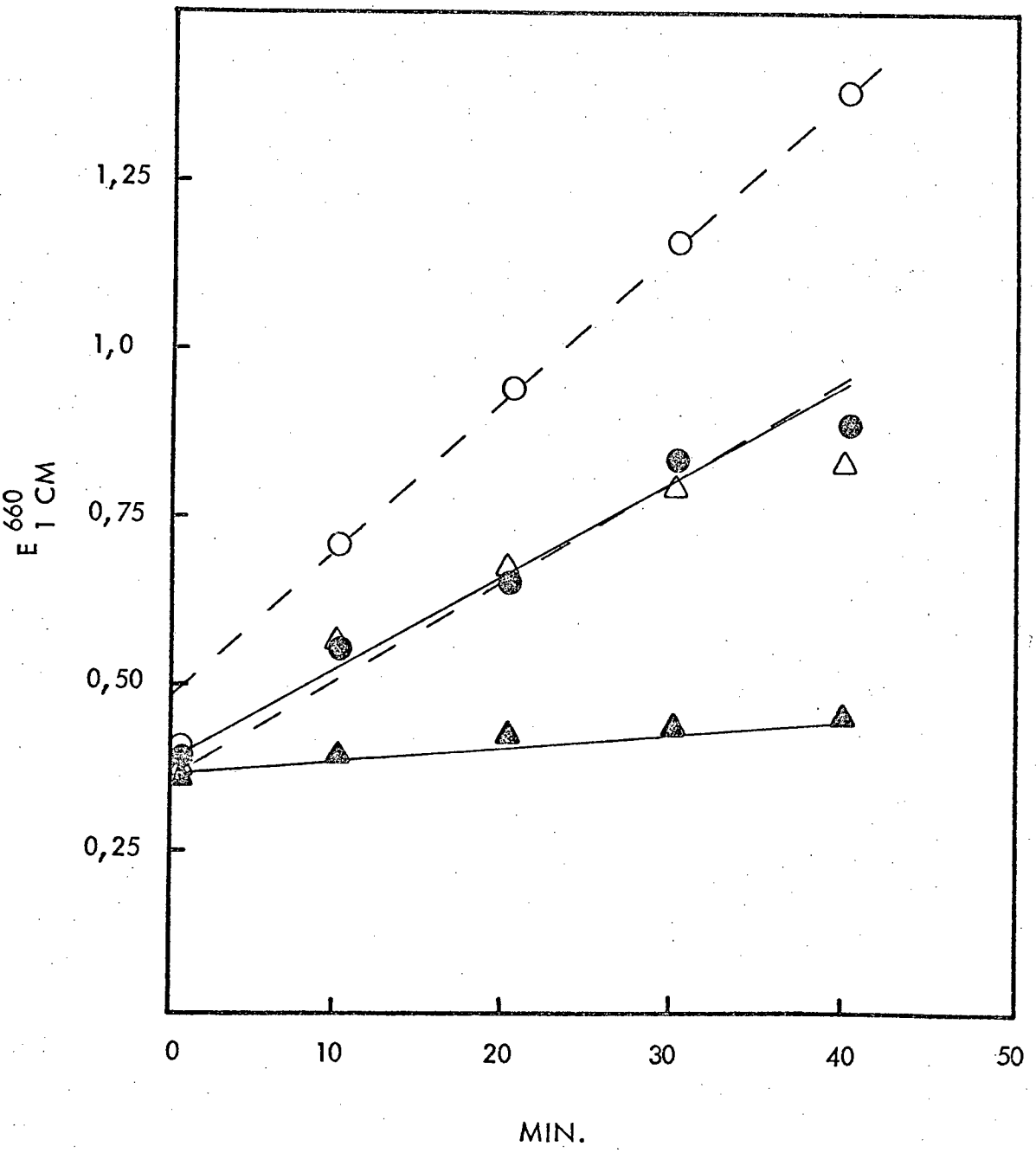


FIG. 18. THE EFFECT OF ADRENALINE ON PHOSPHORYLASE ACTIVITY OF RAT SKELETAL MUSCLE. CONTROL -AMP (▲——▲) CONTROL +AMP (●——●) POST ADRENALINE -AMP (△— —△) AND POST ADRENALINE +AMP (○— —○).

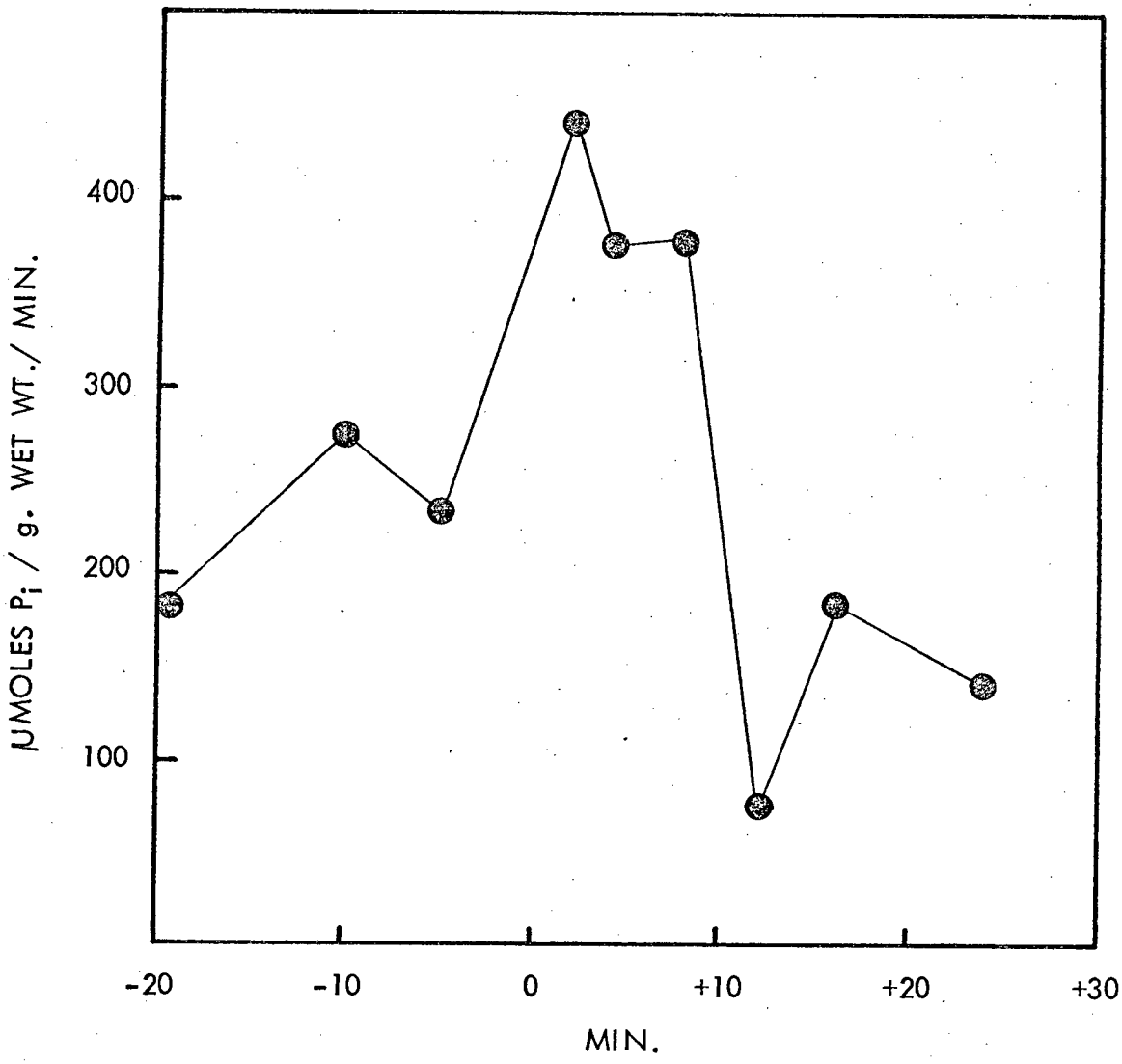


FIG. 19. PIG R101. TOTAL SKELETAL MUSCLE PHOSPHORYLASE ACTIVITY (DETERMINED IN THE PRESENCE OF AMP) DURING THE INDUCTION OF MALIGNANT HYPERTHERMIA.

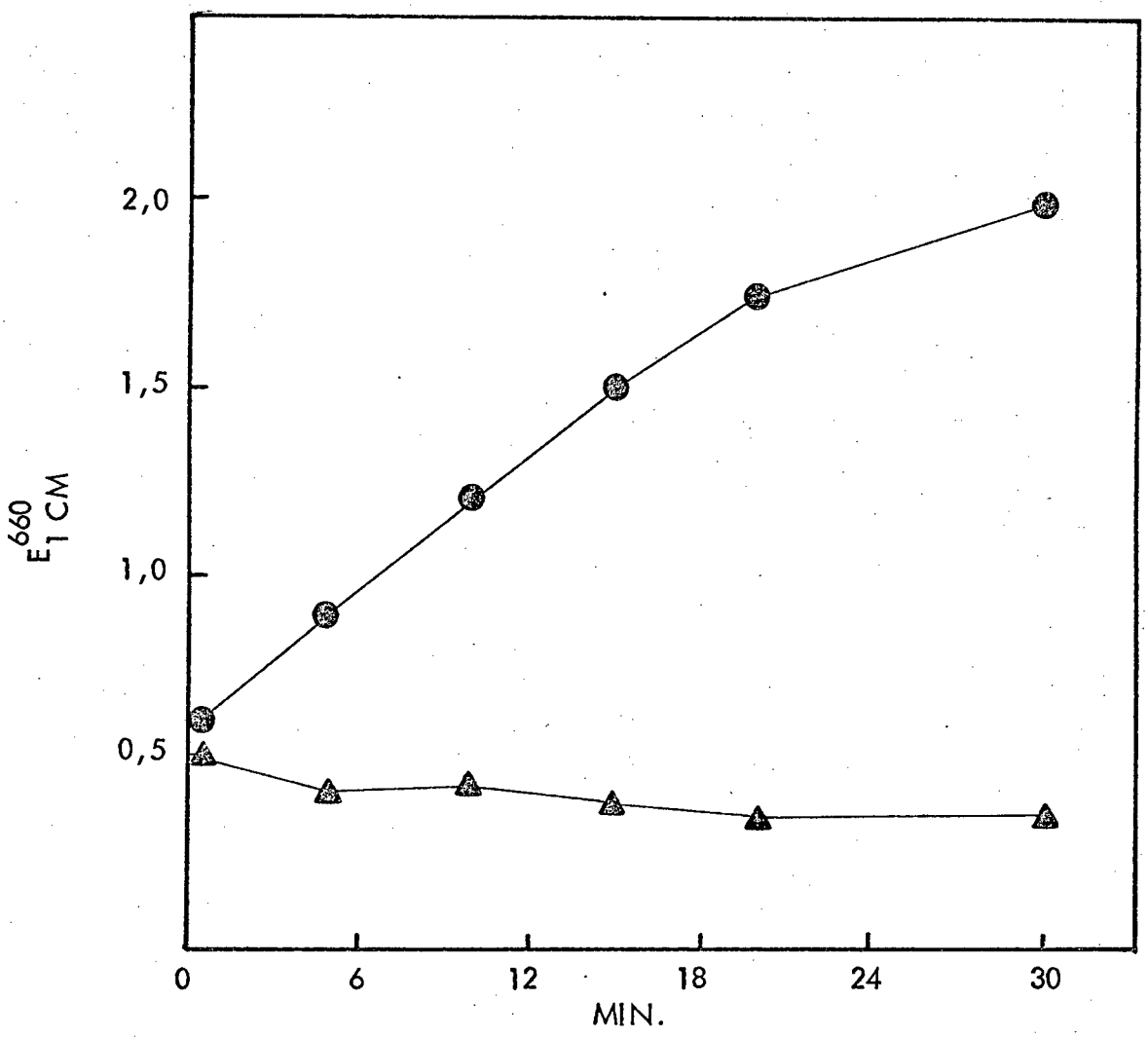


FIG. 20. PIG R101. PHOSPHORYLASE ACTIVITY MEASURED WITH AND WITHOUT AMP, AT -20 MIN. DURING THIOPENTONE ANAESTHESIA. THE PHOSPHOMOLYBDATE COLOUR PRODUCED BY RELEASED P_i FROM G-1-P (SEE DETAILS IN METHODOLOGY) IS PLOTTED ON ABSCISSA AGAINST INCUBATION TIME.

All determinations of pig muscle phosphorylases a and b both in control and hyperthermic animals failed to reveal any phosphorylase activity in the absence of AMP (Fig. 20). In fact, there was a slight drop instead of an increase in P_i as measured by the phosphomolybdate method indicating an absence of phosphorylase a activity. Phosphorylase activities were determined in three pigs; one normal and two hyperthermic pigs. Serial determinations before and after the administration of halothane, amounting to a total of 20 determinations for the three pigs, all showed no P_i release from G-1-P in the absence of AMP under the conditions of the assay.

COMMENT ON RESULTS

Concentrations of all glycolytic intermediates in skeletal muscle rose significantly during succinyl choline-induced acceleration of glycolytic flux. The compounds most affected were glucose-1-phosphate, glucose-6-phosphate, fructose-6-phosphate, fructose-1,6-diphosphate

and α-glycerophosphate, all these being elevated 3 and 10 fold, as compared with control values. 3-Phosphoglycerate and 2-phosphoglycerate were less affected. In Pig 22B and Pig 139, concentrations of 2- and 3-phosphoglycerates rose to approximately twice the starting concentrations. In Pig 137 the concentrations remained unchanged.

Malate, AMP and ADP concentrations increased slightly about 2 to 3 fold of the value at the outset. In Pig 137, the AMP content did not change.

Concentrations of phosphoenolpyruvate and pyruvate were unaltered, except for Pig 139, in which the concentration of phosphoenolpyruvate fell and Pig 137, whose pyruvate concentration rose about 2 fold.

Creatine phosphate in the muscle of Pig 139 fell rapidly from 10 to 2 μmoles/g. wet wt. whilst that of Pig 22B declined only from 11 to 9 μmoles/g. wet wt. In Pig 137, the initial concentration of CP was less and subsequently fell from 5 to 3 μmoles/g. wet wt.

Concentrations of muscle ATP in Pig 137 and Pig 22B were maintained after glycolytic flux had been vastly accelerated. In Pig 139, however, there was a slight fall in ATP concentration, from 4 to 3 μmoles/g. wet wt.

PART IV

D I S C U S S I O N

The disappearance of glycogen from skeletal muscle, the elevation of muscle and plasma lactate, and the rise in blood glucose were observed following the induction of the syndrome, Malignant Hyperthermia, in these pigs. These biochemical changes in the intact animal, which are part of the syndrome, are the consequences of rapid breakdown of muscle glycogen. This fact does not, however, exclude the possibility that reversal of glycolysis in muscle is somewhat impaired or that the rate of hepatic uptake of lactic acid from the blood is restricted. Such phenomena could contribute in part to the elevated concentrations of lactic acid which were observed. Nevertheless, in view of the rapid rate of accumulation of plasma lactate, it is felt that stimulation of glycogen breakdown was the mechanism of paramount importance.

In terms of stoichiometry, muscle glycogen can account for the elevated levels of plasma lactate (20 - 25 mM) attained in this condition. It has also been computed that approximately 50% of the excess heat appearing during Malignant

Hyperthermia can be accounted for in terms of this rapid glycolytic flux (BERMAN and KENCH, 1971).

It would seem obvious to consider if muscle rigor or contraction encountered in this condition also arises from accelerated glycogen breakdown. The exact mechanism by which glycogen catabolism is initiated during normal muscular contraction is still in dispute (pp. 31-33). It would seem, however, that Ca^{++} ions could provide the link between muscular contraction and glycogenolysis. A reversible system for the activation of the phosphorylase step by Ca^{++} has recently been demonstrated (OZAWA, HOSOI and EBASHI, 1967; HEILMEYER, MEYER, HASCHKE and FISCHER, 1970).

Malignant Hyperthermia in human patients can be divided into two groups on the basis of the presence or absence of muscle rigor. Two separate mechanisms may, therefore, be capable of starting this syndrome. Irrespective of the primary stimulus, hyperpyrexia and lactacidosis develop, although the nature of the latter has not been fully elucidated (KALOW, BRITT, TERREAU and HAIST, 1970). All previous animals

studied in this laboratory up till the present, approximately 30, have developed rigor and hyperthermia. Pig 137 described here is the first animal suffering from hyperthermia without rigor which has been described to our knowledge, and he was similar, therefore, to the non-rigorous human patients.

It has been previously shown that rigor is not associated with low concentrations of ATP in the muscle (BERMAN, HARRISON, BULL and KENCH, 1970). Rigor developed in Pigs 22B and 139 whilst ATP levels remained above 4 - 5 µmoles/g. wet weight and in Pig 139 ATP did not fall until just prior to death. In Pig 22B, ATP remained constant. Onset of rigor does not appear to be caused by depletion of ATP. HARRISON, SAUNDERS, BIEBUYCK, HICKMAN, DENT, WEAVER and TERBLANCHE (1969) demonstrated that <u>in vitro</u> the muscle of pigs prone to hyperthermia, as compared with normal pigs, had a tendency towards an abnormal fall in ATP content. This drop was only apparent after prolonged incubation.

Concentrations of creatine phosphate in the muscle were initially maintained in the hyperthermic syndrome, although resting levels in susceptible pigs appeared to be lower than usually recorded in skeletal muscle (15 μmoles/g.wet weight). In Pig 139, however, CP did fall considerably, from 10 to 2 μmoles/g.wet weight.

The trend of creatine phosphate concentration was a downward course as hyperthermia continued, but ATP concentration was not lowered. This was presumably due to resynthesis of ATP by anaerobic glycolysis, at the expense of ADP. If this is true, ADP may not be the crucial stimulator of glycogenolysis. ADP levels did not, in fact, rise significantly as would be expected if the nucleotide was responsible for activation of phosphofructokinase.

AMP followed the same trend as ADP, and stayed constant in Pig 137, and rose only slightly in Pigs 22B and 139. AMP, like ADP, could not, therefore, be envisaged as an activator of the phosphofructokinase step, nor be responsible for acceleration of the phosphorylase step, nor be responsible for acceleration of the phosphorylase step by functioning as an activator

of phosphorylase b: unless, that is, AMP is present in small discrete pools in high concentration in the locality of these enzymes, the contribution of such minute metabolic backwaters being too small to be appreciated in measurements of overall cellular AMP.

Individual glycolytic intermediates were assayed and the ratios of intermediate pairs were calculated in order to locate the control points in this system. The glycolytic flux was accompanied by elevation of most of the glycolytic intermediates. There was a slight increase in the ratio $\frac{[2\text{-PG}]}{[\text{PEP}]}$ in Pigs 22B and 139, pointing to the enolase step as slightly rate-limiting. In Pigs 22B and 137, there was an increase in the ratio $\frac{[\text{PEP}]\,[\text{ADP}]}{[\text{PYR}]\,[\text{ATP}]}$ suggesting that the pyruvate kinase step might be facilitated. This ratio for Pig 139 dropped slightly. The ratios did not approach the values seen during stimulation of anaerobic glycolysis in perfused rat heart in which the ratio of the concentrations of these substrates was dramatically altered and in such a way as could arise from activation of PFK (WILLIAMSON, 1966). The

inference is that no particular control point was stimulated or became rate limiting on the augmented flow of skeletal muscle glycogenolysis in porcine Malignant Hyperthermia.

These observations raise the question whether a simple mechanism, based on the activation of glycogen phosphorylase, could be responsible for the increased glycolytic flux. No conversion of phosphorylase b to a could be demonstrated during the increased catabolism of glycogen. There was also no significant accumulation of AMP which could account for the in vivo activity of the AMP-dependent phosphorylase b form. This mechanism of phosphorylase activation is, in any event, of doubtful physiological significance. The rapid glycogenolysis and lactate accumulation which occurs post-mortem in some pigs has been proven to be associated with the development of a pale, soft exudative (PSE) condition in the skeletal muscle. A pattern of changes in glycolytic intermediates, similar to that obtaining in hyperthermia, has been demonstrated in muscle of animals which, after death, exhibit P.S.E. (KASTENSCHMIDT, HOEKSTRA and BRISKEY, 1968) Phosphorylase b

to a conversion was also not demonstrable in conditions prevailing in P.S.E. (BERMAN and KENCH, UNPUBLISHED OBSERVATIONS).

The techniques employed to demonstrate possible b to a conversion appear to be valid, since, in the present study, it was shown that adrenaline stimulation *in vivo* induced 48% and 66% conversion of phosphorylase b to a in rat and pig skeletal muscle respectively. It is conceivable that change of phosphorylase b to a occurred *in vivo*, but that inactive phosphorylase b was regenerated by phosphorylase phosphatase, an enzyme in skeletal muscle known to be particularly active during isolation procedures.

Activation of a muscle glycogen particle by Ca^{++} has been studied by HEILMEYER, MEYER, HASCHKE and FISCHER, 1970. They observed that the addition of Mg-ATP and Ca^{++} caused an immediate activation of phosphorylase b to a, referred to as the "flash activation" of phosphorylase. It is this rapidly reversible ionic activation by Ca^{++} which makes it such a particularly suitable mechanism for rapid control of metabolic requirements of muscle cells. There is evidence

that Ca^{++} is liberated from intracellular binding sites in skeletal muscle during onset of Malignant Hyperthermia. BERMAN, HARRISON, BULL and KENCH (1970) have demonstrated a rapid rise in plasma Ca^{++} during development of Malignant Hyperthermia.

Ca^{++} accumulation by sarcoplasmic reticulum of rigid and of non-rigid patients who recovered after an episode of Malignant Hyperthermia has been investigated by KALOW, BRITT, TERREAU and HAIST (1970). Ca^{++} uptake by the sarcoplasmic reticulum of the non-rigid patient was normal, but that of the rigid patients was diminished. There appears to be some defect in the sarcoplasmic reticulum activity of these patients. HARRISON (1970) has shown that the syndrome of Malignant Hyperpyrexia in susceptible Landrace pigs can be prevented by treatment with procaine, which is known to prevent caffeine-induced rigor which accrues from inhibition of rebinding of Ca^{++} to the sarcoplasmic reticulum. Release of Ca^{++} would serve to activate a myosin-ATP-ase and the contractile mechanism and also to provoke rigor.

The above arguments are based upon absence of changes in concentration of factors known to

control glycolytic flux. The concentrations of ATP and other cofactors determined in this study were those of the whole tissue, and one cannot exclude, at this time, that local accumulation of ADP within the relevant cellular compartments could have accelerated glycogenolysis.

From the present study, a sequence of events during development of the syndrome Malignant Hyperpyrexia has emerged. The disease is based on a genetic susceptibility to the effects of succinyl choline or halothane. These anaesthetic agents appear to interfere with the binding capacity of Ca^{++} by the sarcoplasmic reticulum. The defective sarcoplasmic reticulum releases Ca^{++} into the sarcoplasm, where, in turn, it stimulates glycogen phosphorylase, activates the contractile mechanism resulting in rigor or contracture of skeletal muscle and stimulates Ca^{++}-activated Myosin-ATPase. The rapid breakdown of ATP is offset by resynthesis from phosphate and from increased glycolytic rate. When these sources of supply become depleted, concentrations of ATP fall.

PART V

S U M M A R Y

Skeletal muscle metabolism in the syndrome, Malignant Hyperpyrexia, induced by the anaesthetic agents halothane or succinyl choline has been studied. The most outstanding biochemical feature of this syndrome is the development of severe systemic lactacidosis, due to the accelerated breakdown of skeletal muscle glycogen. This accompanies the development of hyperthermia, and rigidity or rigor of the limbs.

In order to establish the origin of the lactacidosis and mechanism of activation of glycogenolysis, the glycolytic intermediates of skeletal muscle have been measured. There is a rise in all the glycolytic intermediates; some more than others. The most affected were glucose-1-phosphate, glucose-6-phosphate, fructose-6-phosphate, fructose-1,6-diphosphate and ∝-glycerophosphate. ATP levels remained constant and only dropped prior to death. The ATP levels are thus maintained by the increased glycolytic flux. Creatine phosphate levels were variable but fell if pre-rigor levels were not depressed. Muscle ∝-glycerophosphate, malate and lactate content were all increased suggesting that NADH is accumulating in the cytosol.

The mass/action ratios of the glycolytic intermediates have been calculated in order to locate the control points in this system. The ratios $[2PG]/[PEP]$ and $[PEP][ADP]/[PYR][ATP]$ were increased suggesting that the enolase step was slightly rate limiting and the pyruvate kinase step was activated; the latter possibly by increased availability of ADP although total muscle ADP concentration did not increase significantly. The phosphofructokinase step did not appear to be specifically activated.

It is postulated that accelerated glycogenolysis is due to activation of the phosphorylase reaction and that intermediates accumulate due to subsequent rate limiting steps.

No phosphorylase b to a conversion could be detected during increased glycogenolysis but it is suggested that this activation does occur by a "flash activation" induced by liberation of Ca^{++} from intracellular binding sites possibly consequent to a defect in uptake of Ca^{++} by the sarcoplasmic reticulum which follows halothane or succinyl choline in the susceptible animal.

Release of Ca^{++} can thus account for enhanced

myofibrillar ATPase activity, rigor and acceleration of muscle glycogenolysis by activation of phosphorylase similar to the coordination of these processes which occur during physiological muscle activity.

PART VI

R E F E R E N C E S

A

ADAM, H. (1965) Adenosine-5'-diphosphate and adenosine-5'-monophosphate. Methods of Enzymatic Analysis, ed. by H.U. Bergmeyer, New York and London, pp. 573 - 577.

ATKINSON, D.E. and WALTON, G.M. (1965). Kinetics of regulatory enzymes. Escherichia coli phosphofructokinase. J. Biol. Chem., 240, 757 - 763.

B

BARANOWSKI, T., ILLINGWORTH, B., BROWN, D.H. and CORI, F. (1957). The isolation of pyridoxal-5'-phosphate from crystalline muscle phosphorylase. Biochim. Biophys. Acta, 25, 16 - 21.

BATTELL, M.L., ZARKADAS, C.G., SMILLIE, L.B. and MADSEN, N.B. (1968) The sulphydryl groups of muscle phosphorylase. Identification of cysteinyl peptides related to function. J. Biol. Chem., 243, 6202 - 6209.

BELOCOPITOW, E., APPLEMAN, M.M. and TORRES, H.N. (1965) Factors affecting the activity of muscle glycogen synthetase. Regulation by Ca^{++}. J. Biol. Chem., 240, 3473 - 3478.

BELOCOPITOW, E., FERNANDEZ, M.C.G., BIRNBAUMER, L. and TORRES, H.N. (1967) Factors affecting muscle glycogen synthetase activity. Comparative study of the different dependent forms of glycogen synthetase. J. Biol. Chem., 242, 1227 - 1231.

BERGMEYER, H.U. and KLOTZSCH, H. (1965) D-glucose-1-phosphate. Methods of Enzymatic Analysis, ed. by H.U. Bergmeyer, New York and London, pp. 131 - 133.

BERMAN, M.C., HARRISON, G.G., BULL, A.B. and KENCH, J.E. (1970) Changes underlying halothane-induced Malignant Hyperpyrexia in Landrace pigs. Nature, 225, 653 - 655.

BERMAN, M.C. and KENCH, J.E. (1971) Muscle metabolism in pigs during Malignant Hyperthermia. Proceedings of the International Symposium on Malignant Hyperthermia, Toronto, 1971.

BOYER, P.D., LARDY, H.A. and PHILLIPS, P.H. (1943) Further studies on the role of potassium and other ions in the phosphorylation of the adenylic system. J. Biol. Chem., 149, 529 - 541.

BRITT, B.A. and KALOW, W. (1970). Malignant hyperthermia: Aetiology unknown. Canad. Anaesth. Soc. J., 17, 316 - 330.

BÜCHER, T. and HOHORST, H.J. (1965) Dihydroxyacetone phosphate, fructose-1,6-diphosphate and D-glyceraldehyde-3-phosphate. Determination with glycerol-1-phosphate dehydrogenase, aldolase and triosephosphate isomerase. Methods of Enzymatic Analysis, ed. by H.U. Bergmeyer, New York and London, pp. 246 - 252.

C

CHANCE, B., HOLMES, W., HIGGENS, J. and CONNELLY, C.M. (1958) Localization of interaction sites in multi-component transfer systems: theorems derived from analogues. Nature, 182, 1190 - 1193.

CHELALA, C.A. and TORRES,H.N. (1969) Interconvertible forms of muscle phosphorylase phosphatase. Biochim. Biophys. Acta, 178, 423 - 426.

CORI, G.T. and CORI, C.F. (1945) The enzymatic conversion of phosphorylase a to b. J. Biol. Chem., 158, 321 - 332.

CORI, F., CORI, G.T. and GREEN, A.A. (1943) Crystalline muscle phosphorylase kinetics. J. Biol. Chem., 151, 39 - 55.

CORI, G.T. and GREEN, A.A. (1943) Crystalline muscle phosphorylase. Prosthetic group. J. Biol. Chem., 151, 31 - 38.

CORI, C.F. and ILLINGWORTH, B. (1957) The prosthetic group of phosphorylase. Proc. Natl. Acad. Sci. U.S.A., 43, 547 - 552.

CORI, G.T. and LARNER, J. (1951) Action of amylo-1,6-glucosidase and phosphorylase on glycogen and amylopectin. J. Biol. Chem., 188, 17 - 29.

CORI, C.F., SCHMIDT, G. and CORI, G.T. (1939) The synthesis of a polysaccharide from glucose-1-phosphate in muscle extract. Science, 89, 464 - 465.

CORNBLATH, M., STEINER, D.F., BRYAN, P. and KING, J. (1965) Uridine-diphosphoglucose glucosyltransferase in human erythrocytes. Clin. Chim. Acta, 12, 27 - 32.

COWGILL, R.W. (1959) Lobster muscle phosphorylase: Purification and properties. J. Biol. Chem., 234, 3146 - 3153.

CZOK, R. and ECKERT, L. (1965) D-3-phosphoglycerate, D-2-phosphoglycerate, phosphoenolpyruvate. Determination with phosphoglycerate mutase, enolase, pyruvic kinase and lactic dehydrogenase. Methods of Enzymatic Analysis, ed. by H.U. Bergmeyer, New York and London, pp. 229 - 233.

D

DANFORTH, W.H. (1965) Glycogen synthetase activity in skeletal muscle. Interconversion of two forms and control of glycogen synthesis. J. Biol. Chem., 240, 588 - 593.

DE LANGE, R.J., KEMP, R.G., RILEY, D.W., COOPER, R.A. and KREBS, E.G. (1968) Activation of skeletal muscle phosphorylase kinase by adenosine triphosphate and adenosine 3',5'-monophosphate. J. Biol. Chem., 243, 2200 - 2208.

DRUMMOND, G.I. and DUNCAN, L. (1966) Activation of cardiac phosphorylase b kinase. J. Biol. Chem., 241, 5893 - 5898.

DRUMMOND, G.I. and DUNCAN, L. (1968) On the mechanism of activation of phosphorylase b kinase by calcium. J. Biol. Chem., 243, 5532 - 5538.

DRUMMOND, G.I., DUNCAN, L. and FRIESON, A.J.D. (1965) Some properties of cardiac phosphorylase b kinase. J. Biol. Chem., 240, 2778 - 2785.

DRUMMOND, G.I., DUNCAN, L. and HERTZMAN, E. (1966) Effect of epinephrine on phosphorylase b kinase in perfused rat hearts. J. Biol. Chem., 241, 5899 - 5903.

DRUMMOND, G.I., HARWOOD, J.P. and POWELL, C.A. (1969) Studies on the activation of phosphorylase in skeletal muscle by contraction and by epinephrine. J. Biol. Chem., 244, 4235 - 4240.

E

EDITORIAL (1971) Malignant Hyperpyrexia. Brit. Med. J., 3, 69 - 70.

F

FISCHER, E.H., GRAVES, D.J., CRITTENDEN, E.R.S. and KREBS, E.G. (1959) Structure of the site phosphorylated in the phosphorylase b to a reaction. J. Biol. Chem., 234, 1698 - 1704.

FISCHER, E.H., GRAVES, D.J. and KREBS, E.G. (1957) Phosphopeptides from ^{32}P-labelled phosphorylase a. Fed. Proc., 16, 180.

FISCHER, E.H. and KREBS, E.G. (1955) Conversion of phosphorylase b to phosphorylase a in muscle extracts. J. Biol. Chem., 216, 121 - 132.

FISCHER, E.H. and KREBS, E.G. (1958) The isolation and crystallization of rabbit skeletal muscle phosphorylase b. J. Biol. Chem., 231 65 - 71.

FISCHER, E.H. and KREBS, E.G. (1966) Relationship of structure to function of muscle phosphorylase. Fed.Proc., 25, 1511 - 1520.

FRIEDMAN, L. and LARNER, J. (1963) Studies on UDPG-α-glucan transglucosylase. Interconversion of two forms of muscle UDPG-α-glucan transglucosylase by a phosphorylation-dephosphorylation reaction sequence. Biochemistry, 2, 669 - 675.

FRIEDMAN, D.L. and LARNER, J. (1965) Studies on uridine diphosphate glucose: ∝-1,4-glucan ∝-4-glucosyltransferase. Catalysis of the phosphorylation of muscle phosphorylase and transferase by separate enzymes. Biochemistry, 4, 2261 - 2264.

G

GARLAND, P.B., RANDLE, P.J. and NEWSHOLME, E.A. (1963) Citrate as an intermediate in the inhibition of phosphofructokinase in rat heart muscle by fatty acids, ketone bodies, pyruvate, diabetes and starvation. Nature, 200, 169 - 170.

GEVERS, W. and DOWDLE, E. (1963) The effect of pH on glycolysis in vivo. Clin. Sci., 25, 343 - 349.

GIORGI, P.P. (1970) Polyamines and amino acid incorporation in vitro into microsomes of rat cerebral cortex. Biochem. J., 120, 643 - 651.

GRAVES, D.J., FISCHER, E.H. and KREBS, E.G. (1960) Specificity studies on muscle phosphorylase phosphatase. J. Biol. Chem., 235, 805 - 809.

GREEN, A.A. and CORI, G.T. (1943) Crystalline muscle phosphorylase. Preparation, properties and molecular weight. J. Biol. Chem., 151, 21 - 29.

H

HAMMERMEISTER, K.E., YUNIS, A.A. and KREBS, E.G. (1965) Studies on phosphorylase activation in the heart. J. Biol. Chem., 240, 986 - 991.

HANABUSA, K. and KOBAYASHI, H. (1967) Phosphorylase kinase in human muscle. J. Biochem., (Tokyo) 61, 662 - 664.

HARRISON, G.G. (1971) Anaesthetic-induced malignant hyperpyrexia: A suggested method of treatment. Brit. Med. J., 3, 454 - 456.

HARRISON, G.G., BIEBUYCK, J.F., TERBLANCHE, J., DENT, D.M., HICKMAN, R. and SAUNDERS, S.J. (1968) Hyperpyrexia during anaesthesia. Brit. Med. J., 3, 594 - 595.

HARRISON, W.H., BOYER, P.D. and FALCONE, A.B. (1955) The mechanism of enzymic phosphate transfer reactions. J. Biol. Chem., 215, 303 - 317.

HARRISON, G.G., SAUNDERS, S.J., BIEBUYCK, J.F., HICKMAN, R., DENT, D.M., WEAVER, V. and TERBLANCHE, J. (1969) Anaesthetic-induced malignant hyperpyrexia and a method for its prediction. Brit. J. Anaesth., 41, 844 - 855.

HASCHKE, R.H., HEILMEYER, L.M.G., MEYER, F. and FISCHER, E.H. (1970) Control of phosphorylase activity in a muscle glycogen particle. Regulation of phosphorylase phosphatase. J. Biol. Chem., 245, 6657 - 6663.

HEDESKOV, C.J., ESMANN, V. and ROSELL-PEREZ, M. (1966) Glycogen content and UDPG-glucan glycosyl transferase activity of normal human lymphocytes. Bioch. Bioph. Acta, 130, 393 - 400.

HEILMEYER, L.M.G., MEYER, F., HASCHKE, H. and FISCHER, E.H. (1970) Control of phosphorylase activity in a muscle glycogen particle. Activation by calcium. J. Biol. Chem., 245, 6649 - 6656.

HERS, H.G. (1964) Glycogen storage disease. Advances in Metabolic Disorders, 1, 1 - 44, ed. by R. Levine and R. Luft, New York and London.

HOFER, H.W. and PETTE, D. The role of the nucleic acid in phosphofructokinase as an "enzyme modulator". Life Sciences, 5, 199 - 204.

HOHORST, H.J. (1965 (a)) L-(+)-Lactate. Determination with lactic dehydrogenase and DPN. Methods of Enzymatic Analysis, ed. by H.U. Bergmeyer, New York and London, pp. 266 - 270.

HOHORST, H.J. (1965 (b)) L-(-)-Malate. Determination with malic dehydrogenase and DPN. Methods of Enzymatic Analysis, ed. by H.U. Bergmeyer, New York and London, pp. 328 - 334.

HOHORST, H.J. (1965 (c)) L-(-)-Glycerol-1-phosphate. Determination with glycerol-1-phosphate dehydrogenase. Methods of Enzymatic Analysis, ed. by H.U. Bergmeyer, New York and London, pp. 215 - 219.

HUIJING, F., NUTTALL, F.G., VILLAR-PALASI, C. and LARNER, J. (1969) UDPGlucose: α-1,4-glucanα-4-glucosyltransferase in heart regulation of the activity of the transferase in vivo and in vitro in rat. A dissociation in the action of insulin on transport and on transferase conversion. Biochim. Biophys. Acta, 177, 204 - 212.

HUSTON, R.B. and KREBS, E.G. (1968) Activation of skeletal muscle phosphorylase kinase by calcium. Identification of the kinase activating factor as a protective enzyme. Biochemistry, 7, 2116 - 2122.

HYVÄRINEN, A. and NIKKILÄ, E.A. (1962) Specific determination of blood glucose with o-toluidine. Clin. Chim. Acta, 7, 140 - 143.

K

KACHMAR, J.F. and BOYER, P.D. (1953) Kinetic analysis of enzyme reactions. The potassium activation and calcium inhibition of pyruvic phosphoferase. J. Biol. Chem., 200, 669 - 682.

KALOW, W., BRITT, B.A., TERREAU, M.E. and HAIST, C. (1970) Metabolic error of muscle metabolism after recovery from Malignant Hyperthermia. Lancet, 11, 895 - 898.

KARPATKIN, S. and LANGER, R.M. (1969) Human platelet phosphorylase. Biochim. Biophys. Acta, 185, 350 - 359.

KASTENSCHMIDT, L.L., HOEKSTRA, W.G. and BRISKEY, E.J. (1968) Glycolytic intermediates and co-factors in "fast"- and "slow-glycolyzing" muscles of the pig. J. Food Sci., 33, 151 - 158.

KELLER, P.J. and CORI, G.T. (1953) Enzymic conversion of phosphorylase a to phosphorylase b. Biochim. Biophys. Acta, 12, 235 - 238.

KELLER, P.J. and CORI, G.T. (1955) Purification and properties of the phosphorylase-rupturing enzyme. J. Biol. Chem., 214, 127 - 134.

KELLER, P.J. and FRIED, M. (1955) Inhibition of the phosphorylase-rupturing enzyme by some trypsin substrates. J. Biol. Chem., 214, 143 - 148.

KEMP, R.G. (1969) Allosteric properties of muscle phosphofructokinase. Binding of Mg ATP to the inhibitory site. Biochemistry, 8, 3162 - 3168.

KEMP, R.G. and FOREST, P.B. (1968) Reactivity of the sulfhydryl groups of phosphofructokinase. Fed. Proc., 27, 456.

KEMP, R.G. and KREBS, E.G. (1967) Binding of metabolites by phosphofructokinase. Biochemistry, 6, 423 - 434.

KENT, A.B., KREBS, E.G. and FISCHER, E.H. (1958) Properties of crystalline phosphorylase b. J. Biol. Chem., 232, 549 - 558.

KLEPPE, K. and DAMJANOVICH, S. (1969) Studies of the SH groups of phosphorylase b. Reaction with 5,5'-dithiobis-(2-nitrobenzoic acid). Biochim. Biophys. Acta, 185, 88 - 102.

KLOTZSCH, H. and BERGMEYER, H.U. D-fructose. Methods of Enzymatic Analysis, ed. by H.U. Bergmeyer, New York and London, pp. 156 - 159.

KREBS, E.G. (1954) The effect of salmine on the activity of phosphorylase. Biochim. Biophys. Acta, 15, 508 - 515.

KREBS, H.A. and EGGLESTON, L.V. (1965) The role of pyruvate kinase in the regulation of gluconeogenesis. Biochem. J., 94, 3C - 4C.

KREBS, E.G. and FISCHER, E.H. (1955) Phosphorylase activity of skeletal muscle extracts. J. Biol. Chem., 216, 113 - 120.

KREBS, E.G. and FISCHER, E.H. (1956) The phosphorylase b to a converting enzyme of rabbit skeletal muscle. Biochim. Biophys. Acta, 20, 150 - 157.

KREBS, E.G., GRAVES, D.J. and FISCHER, E.H. (1959) Factors affecting the activity of muscle phosphorylase b kinase. J. Biol. Chem., 234, 2867 - 2873.

KREBS, E.G., KENT, A.B. and FISCHER, E.H. (1958) The muscle phosphorylase b kinase reaction. J. Biol. Chem., 231, 73 - 83.

KREBS, E.G., LOVE, D.S., BRATVOLD, G.E., TRAYSER, A., MEYER, W.L. and FISCHER, E.H. (1964) Purification and properties of rabbit skeletal muscle phosphorylase b kinase. Biochemistry, 3, 1022 - 1033.

KRZANOWSKI, J. and MATSCHINSKY, F.M. (1969) Regulation of phosphofructokinase by phosphocreatine and phosphorylated glycolytic intermediates. Biochem. Biophys. Res. Commun., 34, 816 - 823.

KUDO, A. and SHUKUYA, R. (1964) Sulfhydryl group of muscle phosphorylase. J. Biochem. (Tokyo), 55, 254 - 259.

L

LAMPRECHT, W. and STEIN, P. (1965) Creatine phosphate. Methods of Enzymatic Analysis, ed. by H.U. Bergmeyer, New York and London, pp. 610 - 616.

LARDY, H. and ZIEGLER, J.A. (1945) The enzymatic synthesis of phosphopyruvate from pyruvate. J. Biol. Chem., 159, 343 - 351.

LING, K.H., MARCUS, F. and LARDY, H.A. (1965) Purification and some properties of rabbit skeletal muscle phosphofructokinase. J. Biol. Chem., 240, 1893 - 1899.

LELOIR, L.F. and CARDINI, C.E. (1957) Biosynthesis of glycogen from uridine diphosphate glucose. J. Amer. Chem. Soc., 79, 6340 - 6341.

LOWRY, O.H., PASSONNEAU, J.V., HASSELBERGER, F.X. and SCHULZ, D.W. (1964) Effect of ischemia on known substrates and cofactors of the glycolytic pathway in brain. J. Biol. Chem., 239, 18 - 30.

M

MADSEN, N.B. and CORI, C.F. (1956) The interaction of muscle phosphorylase with p-chloromercuribenzoate. Inhibition of activity and effect on the molecular weight. J.Biol. Chem., 223, 1055 - 1065.

MADSEN, N.B. and CORI, C.F. (1957) The binding of adenylic acid by muscle phosphorylase. J. Biol. Chem., 224, 899 - 908.

MADSEN, N.B. and GURD, F.R.N. (1956) The interaction of muscle phosphorylase with p-chloromercuribenzoate. The reversible dissociation of phosphorylase. J. Biol. Chem., 223, 1075 - 1087.

MANSOUR, T.E. (1962) Effect of serotonin on glycolysis in homogenates from the liver fluke Fasciola hepatica. J. Pharm. Expt. Therap., 135, 94 - 101.

MANSOUR, T.E. (1963) Studies on heart phosphofructokinase: Purification, inhibition and activation. J. Biol. Chem., 238, 2285 - 2292.

MANSOUR, T.E. (1965) Studies on heart phosphofructokinase. Active and inactive forms of the enzyme. J. Biol. Chem., 240, 2165 - 2172.

MANSOUR, T.E., CLAGUE, M.E. and BEERNINK K.D. (1962) Effect of cyclic 3, 5-AMP on heart phosphofructokinase. Fed Proc., 21, 238.

MANSOUR T.E. and MANSOUR J.M. (1962) Effects of serotonin (5-hydroxy tryptamine) and adenosine 3'-5'-phosphate on phosphofructokinase from the liver fluke Fasciola hepatica. J. Biol. Chem., 237, 629 - 634.

MANSOUR, T.E. WAKID, N. and SPROUSE H.M. (1966) Studies on heart phosphofructokinase. Purification, crystallization and properties of sheep heart phosphofructokinase. J. Biol. Chem., 241, 1512 - 1521.

McARDLE, B. (1951) Myopathy due to a defect in muscle glycogen breakdown. Clin. Sci., 10, 13 - 35.

MERLEVEDE, W., GORIS, J. and DE BRANDT, C. (1969) Interconversion *in vitro* of two forms of liver phosphorylase phosphatase. European J. Biochem., 11, 499 - 502.

MERLEVEDE, W. and RILEY, G.A. (1966) The activation and inactivation of phosphorylase phosphatase from bovine adrenal cortex. J. Biol. Chem., 241, 3517 - 3524.

METZGER, B.E., GLASER, L. and HELMREICH, E. (1968) Purification and properties of frog skeletal muscle phosphorylase. Biochemistry, 7, 2021 - 2036.

MEYER, W.L., FISCHER, E.H. and KREBS, E.G. (1964) Activation of skeletal muscle phosphorylase b kinase by Ca^{++}. Biochemistry, 3, 1033 - 1039.

MEYER, F., HEILMEYER, L.M.G., HASCHKE, H. and FISCHER, E.H. (1970) Control of phosphorylase activity in a muscle glycogen particle. Isolation and characterization of the protein-glycogen complex. J. Biol. Chem., 245, 6642 - 6648.

MILDVAN, A.S. and COHEN, M. (1965) Kinetic and magnetic resonance studies of the pyruvate kinase

reaction. Divalent metal complexes of pyruvate kinase. J. Biol. Chem., 240, 238 - 246.

MIYAMOTO, E., KUO, J.F. and GREENGARD, P. (1969) Adenosine-3',5',monophosphate dependent protein kinase from brain. Science, 165, 63 - 65.

MURPHY, T.A. and WYATT, G.R. (1965) The enzymes of glycogen and trehalose synthesis in silk moth fat body. J. Biol. Chem., 240, 1500 - 1508.

N

NAMM, D.H. and MAYER, S.E. (1967) Effects of epinephrine on cardiac cyclic AMP, phosphorylase activation and contractility. Fed. Proc., 26, 351.

NOLAN, C., NOVOA, W.B., KREBS, E.G. and FISCHER, E.H. (1964) Further studies on the site phosphorylated in the phosphorylase b to a reaction. Biochemistry, 3, 541 - 551.

O

ONCLEY, J.L. (1943) Note on the molecular weight of cyrstalline phosphorylase. J. Biol. Chem., 151, 27 - 28.

ÖZAND, P. and NARAHARA, H.T. (1964) Regulation of glycolysis in muscle. Influence of insulin, epinephrine and contraction on phosphofructokinase activity in frog skeletal muscle. J. Biol. Chem., 239, 3146 - 3152.

OZAWA, E., HOSOI, K. and EBASHI, S. (1967) Reversible stimulation of muscle phosphorylase b kinase by low concentrations of Ca ions. J. Biochem. (Tokyo), 61, 531 - 533.

P

PARMEGGIANI, A. and BOWMAN, R.H. (1963) Regulation of phosphofructokinase activity by citrate in normal and diabetic muscle. Biochem. Biophys. Res. Commun., 12, 268 - 273.

PARMEGGIANI A. and KREBS, E.G. (1965) Crystallization of rabbit muscle phosphofructokinase. Biochem. Biophys. Res. Commun., 19, 89 - 94.

PARMEGGIANI, A., LUFT, J.H., LOVE, D.S. and KREBS, E.G. (1966) Crystallization and properties of rabbit skeletal muscle phosphofructokinase. J. Biol. Chem., 241, 4625 - 4635.

PASSERON, H., DE ASUA, L.J. and CARMINATTI, H. (1967) Fructose-1,6-diphosphate, a reactivator of Cu^{++}-inhibited pyruvate kinase from liver. Biochem. Biophys. Res. Commun., 27, 33 - 38.

PASSONNEAU, J.V. and LOWRY, O.H. (1962) Phosphofructokinase and the Pasteur effect. Biochem. Biophys. Res. Commun., 7, 10 - 15.

PASSONNEAU, J.V. and LOWRY, O.H. (1963) Phosphofructokinase and the control of the citric acid cycle. Biochem. Biophys. Res. Commun., 13, 372 - 379.

PHILIP, G. and GRAVES, D.J. (1968) Dinitrophenylation of glycogen phosphorylase. Preparation

and properties of active dinitrophenyl derivatives. Biochemistry, 7, 2093 - 2101.

PIRAS, R., ROTHMAN, L.B. and CABIB, E. (1967) Metabolite regulation of the I and D form of rat muscle glycogen synthetase. Biochem. Biophys. Res. Commun., 28, 54 - 58.

POGSON, C.I. (1968 (a)). Two interconvertible forms of pyruvate kinase in adipose tissue. Biochem. Biophys. Res. Commun., 30, 297 - 302.

POGSON, C.I. (1968 (b)) Adipose tissue pyruvate kinase. Properties and interconversion of two active forms. Biochem. J., 110, 67 - 77.

POSNER, J.B., HAMMERMEISTER, K.E., BRATVOLD, G.E. and KREBS, E.G. (1964) The assay of adenosine-3',5'-phosphate in skeletal muscle. Biochemistry, 3, 1040 - 1044.

POSNER, J.B., STERN, R. and KREBS, E.G. (1965) Effects of electrical stimulation and epinephrine on muscle phosphorylase, phosphorylase b kinase, and adenosine 3',5'-phosphate. J. Biol. Chem., 240, 982 - 985.

R

RALL, T.W., WOSILAIT, W.D. and SUTHERLAND, E.W. (1956) The interconversion of phosphorylase a and phosphorylase b from dog heart muscle. Biochim. Biophys. Acta, 20, 69 - 76.

REYNARD, A.M., HASS, L.F., JACOBSEN, D.D. and BOYER, P.D. (1961) The correlation of reaction kinetics and substrate binding with the mechanism of pyruvate kinase. J. Biol. Chem., 236, 2277 - 2283.

RILEY, D.W., DE LANGE, R.J., BRATVOLD, G.E. and KREBS, E.G. (1968) Reversal of phosphorylase kinase activation. J. Biol. Chem., 243, 2209 - 2215.

ROSELL-PEREZ, M. and LARNER, J. (1964 (a)). Studies on UDPG-∝-glucan transglucosylase. Purification and characterization of two forms from rabbit skeletal muscle. Biochemistry, 3, 75 - 81.

ROSELL-PEREZ, M. and LARNER, J. (1964 (b)). Studies on UDPG-∝-glucan transglucosylase. Two forms of the enzyme in dog skeletal muscle and and their interconversion. Biochemistry, 3, 81 - 88.

ROSELL-PEREZ, M., VILLAR-PALASI, C. and LARNER, J. (1962) Studies on UDPG-glycogen transglucosylase. Preparation and differentiation of two activities of UDPG-glycogen transglucosylase from rat skeletal muscle. Biochemistry, 1, 763 - 768.

SACKTOR, B., WILSON, J.E. and TIEKERT, C.G. (1966) Regulation of glycolysis in brain, in situ, during convulsions. J. Biol. Chem., 241, 5071 - 5075.

SACKTOR, B. and WORMSER-SHAVIT, E. (1966) Regulation of metabolism in working muscle in vivo. Concentrations of some glycolytic tricarboxylic acid cycle, and amino acid intermediates in insect flight muscle during flight. J. Biol. Chem., 241, 624 - 631.

SALAS, M.L., SALAS, J. and SOLS, A. (1968) Desensitization of yeast phosphofructokinase to ATP inhibition by treatment with trypsin. Biochem. Biophys. Res. Commun., 31, 461 - 466.

SCHEUER, J. and BERRY, M.N. (1967) Effect of alkalosis on glycolysis in the isolated rat heart. Am. J. Physiol., 213, 1143 - 1148.

SEERY, V.L.L., FISCHER, E.H. and TELLER, D.C. (1967) A reinvestigation of the molecular weight of glycogen phosphorylase. Biochemistry, 6, 3315 - 3327.

SOLVONUK, P.F. and COLLIER, H.B. (1955) The pyruvic phosphoferase of erythrocytes. Properties of the enzyme and its activity in erythrocytes of various species. Canad. J. Bioch. Phys., 33, 38 - 45.

STEINMETZ, M.A. and DEAL, W.C. (1966) Metabolic control and structure of glycolytic enzymes. Dissociation and subunit structure of rabbit muscle pyruvate kinase. Biochemistry, 5, 1399 - 1405.

SUTHERLAND, E.W. and WOSILAIT, W.D. (1956) The relationship of epinephrine and glucagon to liver phosphorylase. Liver phosphorylase; preparation and properties. J. Biol. Chem., 218, 459 - 468.

T

TANAKA, T., HARANO, Y., MORIMURA,H. and MORI, R. (1965) Evidence for the presence of two types of pyruvate kinase in rat liver. Biochem. Biophys. Res. Commun., 21, 55 - 60.

TANAKA, T., SUE, F. and MORIMURA, H. (1967). Feed-forward activation and feed-back inhibition of pyruvate kinase type L of rat liver. Biochem. Biophys. Res. Commun., 29, 444 - 449.

TÁRNOKY, K. and NAGY, S. (1963) Spectrophotometric determination of glycogen with o-toluidine. Clin. Chim. Acta, 8, 627 - 628.

TRAUT, R. and LIPMANN, F. (1963) Activation of glycogen synthetase by glucose-6-phosphate. J. Biol. Chem., 238, 1213 - 1221.

TRIVEDI, B. and DANFORTH, W.H. (1966) Effect of pH on the kinetics of frog muscle phosphofructokinase. J. Biol. Chem., 241, 4110 - 4112.

V

VELICK, S.F. and WICKS, L.F. (1951) The amino acid composition of phosphorylase. J. Biol. Chem., 190, 741 - 751.

VILLAR-PALASI, C. and GAZQUEZ-MARTINEZ, I. (1968) Purification, properties and mechanism of interconversion of kidney phosphorylase. Biochim. Biophys. Acta, 159, 479 - 489.

VILLAR-PALASI, C. and WENGER, J.I. (1967) *In vivo* effect of insulin on muscle glycogen synthetase. Identification of the action pathway. Fed. Proc., 26, 563.

W

WALSH, D.A., PERKINS, J.P. and KREBS, E.G. (1968) An adenosine-3',5'-monophosphate-dependent protein kinase from rabbit skeletal muscle. J. Biol. Chem., 243, 3763 - 3765.

WANG, J.H., HUMNISKI, P.M. and BLACK, W.J. (1968) Effect of polyamines on glycogen phosphorylase. Differential electrostatic interactions and enzymic properties. Biochemistry, 7, 2037 - 2044.

WANG, J.H., TU, J.I. and LO, F.M. (1970) Effect of glucose-6-phosphate on the nucleotide site of glycogen phosphorylase b. A general approach for negative heterotropic interactions. J. Biol. Chem., 245, 3115 - 3121.

WEBER, G., SINGHAL, R.L., STAMM, N.B. and SRIVASTAVA, S.K. (1965) Hormonal induction and suppression of liver enzyme biosynthesis. Fed. Proc., 24, 745 - 754.

WEBER, G., STAMM, N.B. and FISCHER, E.A. (1965) Insulin: Inducer of pyruvate kinase. Science, 149, 65 - 67.

WILLIAMSON, J.R. (1965) Glycolytic control mechanisms. Inhibition of glycolysis by acetate and pyruvate in the isolated perfused rat heart. J. Biol. Chem., 240, 2308 - 2321.

WILLIAMSON, J.R. (1966) Glycolytic control mechanisms. Kinetics of intermediate changes during the aerobic-anoxic transition in perfused rat heart. J. Biol. Chem., 241, 5026 - 5036.

WILLIAMSON, J.R. (1967) Glycolytic control mechanisms. Effects of iodoacetamide and fluoroacetate on glucose metabolism in the perfused rat heart. J. Biol. Chem., 242, 4476 - 4485.

WILLIAMSON, J.R., CHEUNG, W.Y., COLES, H.S. and HERCZEG, B.E. (1967) Glycolytic control mechanisms. Kinetics of glycolytic intermediate changes during electrical discharge and recovery in the main organ of Electrophorus electricus. J. Biol. Chem., 242, 5112 - 5118.

WILSON, R.D., NICHOLS, R.J., DENT,T.E. and ALLEN, C.R. (1966) Disturbances of the oxidative-phosphorylation mechanism as a possible etiological factor in sudden unexplained hyperthermia occurring during anaesthesia. Anaesthesiology, 27, 231 - 232.

WOSILAIT, W.D. and SUTHERLAND, E.W. (1956) The relationship of epinephrine and glucagon to liver phosphorylase. Enzymatic inactivation of liver phosphorylase. J. Biol. Chem., 218, 469 - 481.

Y

YUNIS, A.A., FISCHER, E.H. and KREBS, E.G. (1960) Crystallization and properties of human muscle phosphorylases a and b. J. Biol. Chem., 235, 3163 - 3168.